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CONTENTS OF THIS ISSUE

Steroidal Sapogenins. VII. Survey of Plants for Steroidal Sapogenins and Other Constituents 1
Monroe E. Wall, Merle M. Krider, C. F. Krewson, C. Roland Eddy, J. J. Willaman, D. S. Corell, and *H. S. Gentry*
The *in Vitro* Evaluation of the Antibacterial Activity of Sulfonamides 7
George M. Naimark and *Lesly White*
The Influence of Whole Body X-Irradiation on the Pharmacological Action of Certain Drugs 9
Patrick F. Belcastro, John E. Christian, and *H. George DeKay*
Composition of Gum Terpentine of Pines. XVIII. A Report on *Pinus pungens, P. glabra,* and *P. teocote* 13
N. T. Mirov, P. M. Hoff, Jr., and *L. B. Gordon*
Nitroalkyl Ethyl Carbonates 15
A. Knevel and *C. E. Miller*
A Pharmacological Study of a Crystalline Glycoside of *Caulophyllum thalictroides* 16
H. C. Ferguson and *L. D. Edwards*
The Ultraviolet Spectrophotometric Determination of Official Phenobarbital Preparations 22
Leland N. Mattson
The Antibacterial Activity of an Extract Obtained from *Ascophyllum nodosum* 24
Diamanie D. Vacca and *Robert A. Walsh*
Studies on the Effects of Surface-Active Agents on the Extraction of Crude Drugs 27
Einar Brochmann-Hanssen
Studies on the Chemotherapy of Experimental Schistosomiasis. II. N-(9-Xanthenyl)amides 32
Howard W. Bond and *George W. Luttermoser*
The Newer Anticholinergic Agents. I. Effectiveness as Anhydrotics 35
Arthur G. Zupko and *Leon D. Prokop*
Spectrophotometric Determination of Morphine and of Codeine 39
William A. Clark and *Arthur J. McBay*
A Preliminary Phytochemical Study of *Hippomane mancinella* L. 43
H. J. Schaeffer, W. M. Lauter, and *P. A. Foote*
Microtoxicology. X. Colorimetric Reactions and Optical Crystallographic Properties of Five Synthetic Antispasmodics 46
Thomas J. Haley and *George L. Keenan*
The Infrared Determination of Erythromycin 48
William H. Washburn
A Comparison of the Determination of Gentisic Acid in Blood by Means of the Ferric Ion-Gentisate Complex With and Without the Presence of Ferrous Ions 50
Mary Cass and *John G. Wagner*
The *in Vitro* Antifungal Activity of a Series of Acridine Salts 52
Stanley M. Viscia and *Donald C. Brodie*
Aralkyl Dithiobiuret Analogs of Tibione 54
Samuel B. Britton and *W. L. Nobles*
A Survey of Fungi from the Order *Melanconiales* for Antibiotic Activity 56
Franklin R. Cole and *Raymond W. Vanderwyk*
The Preparation of Some Amides of Dichloroacetaldehyde 59
William D. Easterly and *Joseph P. LaRocca*
The Isolation of a Material Capable of Producing Experimental Lathyrism 61
Harold P. Dupuy and Jordan G. Lee
A Note on the Thermal Instability of 2-Nitrobutanol-1 63
Clifton Eugene Miller
A Note on the Properties of Some Additional Chloral and α,α,β-Trichlorobutyraldehyde Amides 63
Joseph P. LaRocca and *Woodrow R. Byrum*
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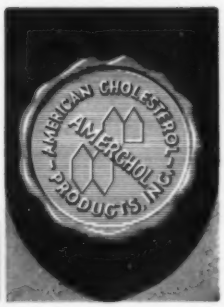

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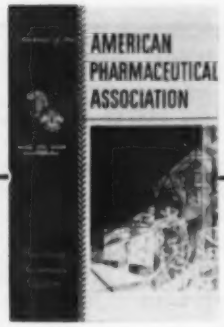

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIII — *JANUARY, 1954* — NUMBER 1 — CONSECUTIVE NO. 2

Steroidal Sapogenins*

VII. Survey of Plants for Steroidal Sapogenins and Other Constituents

By MONROE E. WALL, MERLE M. KRIDER, C. F. KREWSON, C. ROLAND EDDY, J. J. WILLAMAN, D. S. CORELL, and H. S. GENTRY†

The results of the chemical examination of the first 1,000 plant samples received in a survey of plants for steroidal sapogenins are reported. Data are given on 925 samples, representing 292 identified species, 335 unidentified lots, 104 genera, and 30 families. Quantitative data are given for the occurrence of 14 steroidal sapogenins. Qualitative results were obtained for flavonols, alkaloids, tannins, and unsaturated sterols.

CORTISONE was originally isolated from the adrenal cortex. At present commercial cortisone is synthesized from certain bile acids of cattle. Since the supply of these is limited by the number of cattle slaughtered, a cheaper and potentially unlimited plant source of a suitable raw material for the production of this and other therapeutic agents would obviously be of inestimable value. Since 1948, academic, industrial, and governmental research agencies have endeavored to find new and improved starting materials for cortisone.

In 1929, an African species of the genus *Strophanthus*, presumably *S. sarmentosus*, was reported as containing sarmentogenin (1). Since sarmentogenin has an 11-hydroxyl group, it is potentially a much better cortisone precursor than bile acids (2). The National Institutes of Health (NIH), in following up this potential plant source for cortisone, requested the cooperation of the Bureau of Plant Industry, Soils, and Agricultural Engineering in obtaining raw materials of this genus and, in July, 1949, transferred sufficient funds to its Division of Plant Exploration and Introduction (PEI) to support an expedition in West Africa to search for materials of *Strophanthus* species for chemical analysis and propagation. John T. Baldwin, Jr., of the College of William and Mary, was employed to do this exploratory work.

From July, 1949, to June, 1950, Dr. Baldwin made collections of *Strophanthus* and other plants, including *Dioscorea*, in Liberia, Belgian Congo, Gold Coast, Nigeria, British and French

* Received March 16, 1953, from the Eastern Regional Research Laboratory of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Philadelphia, Pa.

† Monroe E. Wall, Merle M. Krider, C. F. Krewson, C. Roland Eddy, and J. J. Willaman are with the Eastern Regional Research Laboratory, Philadelphia, Pa., while D. S. Correll and H. S. Gentry are with the Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture, Beltsville, Md.

In order to expedite the examination of such a large number of plant materials, groups of laboratory workers were organized, each group handling certain phases of the whole procedure. We gratefully acknowledge the work of J. W. Garvin, Walter Rumph, R. A. Pierce, H. M. Neilson, G. H. Eppley, Theodore Perlstein, H. E. Kenney, Arthur Finchler, H. W. Jones, M. L. McClennan, Samuel Serota, R. F. Mininger, H. I. Sinnamon, A. E. Jones, C. S. Fenske, M. K. Scott, M. A. Morris, L. P. Witnauer, A. Turner, Jr., and J. R. Necho.

Cameroons, and French Guinea. In the meantime, additional collections were obtained from various sources. To date, about 75 samples of *Strophanthus* and related genera have been obtained for chemical analysis by NIH, and over 100 collections, including about 25 species, are under propagation. A cooperative agricultural research program on *Strophanthus* with the Federal Experiment Station, Mayaguez, P. R., is now under way to determine the feasibility of growing *Strophanthus* as an agricultural crop. The highest yielding *Strophanthus* material discovered thus far is included in this program.

From the pioneering researches of R. F. Marker and his associates (3) it was known that steroidal sapogenins are readily converted to pregnane compounds with the desirable hormonal side chain. At first only steroids in which the 11-oxygen could be introduced chemically were useful. These included hecogenin with a 12-carbonyl group and diosgenin with unsaturation between carbons 5 and 6 (4, 5). More recently it has been shown that progesterone can be converted microbiologically to 11-α-hydroxy progesterone (6). Hence all progesterone precursors are potentially valuable. Among the steroidal sapogenins, diosgenin is outstanding but sarsasapogenin, smilagenin, and tigogenin can all be used for progesterone synthesis. Sapogenins with hydroxyl groups in positions other than carbon 3 and with 12-carbonyl groups are less satisfactory for this purpose

Marker, *et al.* showed that these sapogenins occur most frequently in plant families in the order Liliales. The several families of greatest interest are Amaryllidaceae, which includes *Agave*, the source of hecogenin; Dioscoriaceae, including primarily the genus *Dioscorea*, the source of diosgenin; and Liliaceae, including (among many other genera) *Yucca*, the source of other sapogenins. It was thus fortunate that Marker's fundamental research served as a preliminary guide as to what to look for and where to look for it.

PROCUREMENT

Following conferences of representatives of the National Institutes of Health, the Bureau of Plant Industry, Soils, and Agricultural Engineering, and the Bureau of Agricultural and Industrial Chemistry (BAIC), (the two latter of the Agricultural Research Administration, U. S. Department of Agriculture), it was decided that the combined forces of the three agencies could make an important contribution to the problem of increasing the cortisone supply. With authorization and funds from Congress, in the fall of 1950, the three agencies initiated their program: PEI to procure raw plant materials for chemical analysis and to try to develop promising species as crops; BAIC to isolate and chemically study potential cortisone precursors in the plant materials procured; NIH to synthesize cortisone from suitable plant steroids isolated by BAIC. Also, NIH was to continue research on *Strophanthus* as originally begun in July, 1949.

In the plant procurement phase, one of us (H. S. Gentry), in September, 1950, started exploration in Mexico and the southwestern United States for *Agave* species, since this region is the center of concentration for this genus. He also collected *Dioscorea* species in central Mexico, as well as a great deal of miscellaneous material. He still continues these North American explorations.

Other collectors who worked with him in Mexico are E. C. Ogden, University of Maine, from September, 1950 to June, 1951, and C. L. Gilly, Michigan State College, from January, 1951 to August, 1951. In addition to this work, Oliver W. Norvell, Carnegie Institution of Washington, explored in Guatemala, Honduras, El Salvador, and Nicaragua. In the meantime, several collaborators obtained materials from various regions in the United States. The principal ones were Robert R. Cruse, who obtained desert plants in the Tucson, Ariz., area; R. K. Godfrey, who obtained yuccas and miscellaneous materials in North and South Carolina; W. H. Duncan, who collected materials in Georgia and Florida; Barton H. Warnock, who obtained valuable materials from the Trans-Pecos region in Texas.

Since South Africa has one of the greatest concentrations of genera and species in the family Amaryllidaceae, Walter H. Hodge, now with PEI, was sent to this region from January to June, 1951. He collected materials from Cape Province, Natal, the Transvaal, and Southern and Northern Rhodesia.

Because of their increasing importance, concentration on collecting *Agave* and *Dioscorea* continues in the hope that the highest-yielding species or strains may be obtained with the idea of getting them into agricultural production as soon as possible. In order to avoid overlooking some other part of the plant kingdom which might give rich yields of sapogenins, plant materials from diverse families outside the Liliales are being chemically tested. These plant samples are not only acquired from nature but are being taken from a rich reservoir of plant materials maintained at the various plant introduction gardens of PEI.

Besides the cooperative agricultural program on *Strophanthus* and, lately, *Dioscorea* with the Federal Experiment Station, Mayaguez, P. R., PEI has a cooperative arrangement with the Firestone Plantations in Liberia to plant, maintain, and test *Strophanthus* in their plantations. Also, an arrangement has been made with the Huntington Botanic Garden, San Marino, Calif., to accept and maintain as a germ plasm center the various species and strains of *Agave* which Dr. Gentry and others are obtaining in the wild. Additional work is being planned at the Experiment Station, Sacaton, Ariz., to learn something of the agricultural possibilities of certain agaves. Besides the above, various tests and preliminary evaluations on *Dioscorea* and other genera are being made at the several plant introduction gardens maintained by PEI.

PROCEDURE

It was first necessary to devise a screening procedure which could be used on large numbers of samples. This procedure had to be as simple as possible, yet yielding correct identification of the sapogenins and, to a certain degree, quantitative information. The method adopted is given below. It was realized early that most of the plants to be submitted would be wild and that hence their chemistry would be either little known or unknown. In order to take advantage of this opportunity, a second procedure was established whereby the samples would be qualitatively examined for six other groups of constituents—flavonols, alkaloids, tannins, sterols, organic acids, and phenols.

The present report covers the first 1,000 accessions. For one reason or another some of these were discarded. Data are given on 925 samples, representing 292 identified and 335 unidentified species,[1] 104 genera, and 29 families. A search through Wehmer (7), Henry (8), Manske and Holmes (9), Chemical Abstracts, Biological Abstracts, and Marker's list (3) disclosed that there is no published chemical information on about half of the species listed. It is hoped, therefore, that the present compilation will prove to be a starting point for plant chemists looking for new constituents and new sources.

DETECTION AND ESTIMATION OF STEROIDS

Full details of the methods used for the detection and estimation of saponins and their derived sapogenins have been presented previously (10, 11). Hence this section will present only the broad outlines of the methods used and the interpretation of the results thus obtained.

Since complete and large-scale macro isolation (11) of steroidal sapogenins is time-consuming, and since many species are negative, all samples received were first put through the much more rapid micro screening procedure (10).

This has three distinct phases: (*a*) detection of saponins, (*b*) isolation of crude sapogenins, and (*c*) estimation of sapogenin content by infrared. A negative finding at any one of these three stages eliminated the sample and only samples positive in all three steps received the macro isolation.

Micro Procedure

Detection of Saponins.—This test and the other subsequent stages of the micro procedure could be carried out with as little as 5 Gm. dry or 20 Gm. wet sample. Where enough sample was available 50 Gm. dry or 200 Gm. wet were extracted with hot 95% alcohol, and made to 1 L.

Practically all saponins, whether steroidal or triterpenoid, have the property of hemolyzing red blood cells. If a 1-ml. aliquot of the above extract did not hemolyze 10 ml. of a saline red blood cell suspension, no significant quantity of steroidal sapogenin could be found by either micro or macro methods. Hence a negative hemolysis test automatically meant that the sample had no steroidal sapogenin but a positive test was not so clean cut.

[1] Subsequent papers in the series will list identifications as they are obtained.

Isolation of Crude Sapogenin.—An aliquot of the extract equivalent to 5.0 Gm. dry sample was concentrated and defatted with benzene. The saponins were hydrolyzed with 4 *N* hydrochloric acid at 75–80° for two hours and the resultant sapogenins extracted with benzene. After acetylation and alkali purification, the crude sapogenin acetate was dried and weighed. A yield of crude acetate less than 10–20 mg. meant less than 0.1% pure sapogenin, and the sample was classified as negative for our purposes and listed thus in this paper.

Infrared Estimation of Sapogenins.—Crude sapogenin acetates were dissolved in carbon disulfide or chloroform and their infrared spectra determined. The following information was thus obtained:

(*a*) Whether steroidal sapogenins were present in the sample from stage 2.

(*b*) If present, whether the content was low (less than 0.1%), moderate (0.1–0.5), or high (greater than 0.5).

(*c*) In favorable cases an indication of the kind and chemical configuration of the sapogenins present (usually when the content was greater than 0.5% and the sample relatively pure).

All samples showing less than 0.1% sapogenin by infrared were classified as negative for our purposes and are thus listed in this paper.

Macro Procedure

Positive samples from stage *c* above were carried through the macro procedure (10). This is essentially a larger-scale and more quantitative isolation, similar in principle to stage *b* above. The crude sapogenins were further purified with solvents, and mixtures were separated by chromatography. The content was determined from the total weight of purified sapogenin. The genins were identified by means of adsorption behavior, melting point, optical rotation, infrared and X-ray diffraction spectra, and if need be by characteristic chemical reactions. Occasionally a sample was found which contained no crystallizable material; such products were also classified as negative.

Hence a sample classified as 0% sapogenin in Table I may have received this rating in the four ways described above.

SCREENING TESTS FOR PLANT CONSTITUENTS OTHER THAN STEROIDS

Preparation of Extracts.—Alcoholic extracts remaining from the hemolytic tests were made ready for qualitative testing for other plant constituents as follows.

Aliquots, in sufficient amounts to give equivalents of 5–10 Gm. dry weight of original plant material, were evaporated to dryness. The residues were each dissolved in 25-ml. portions of hot water and filtered through serological filter pads. This procedure gave solutions representing about 300 mg. of plant per ml. Qualitative tests for alkaloids, tannins, organic acids, and phenols were made upon portions of these water extracts. Tests for sterols were made on chloroform extracts, prepared by dissolving residues from the alcoholic extracts in sufficient chloroform to give concentrations of plant material equivalent to the water extracts. Tests for flavonols were made upon the original alcoholic extracts of the plants.

An arbitrary system of scoring has been used to give a rough estimate of the amounts of the various constituents present, 0 indicating none found; one plus (+) a slight amount present; two plus (++), a moderate amount present; and three plus (+++), a substantial amount.

Flavonols.—The test applied is essentially that used by Willstätter (12) in his early studies on the reduction of quercetin. Beginning with the work of Stein (13) it was found that certain plant constituents gave distinctive color reactions when reduced in alcoholic solution with hydrochloric acid in the presence of metals such as sodium amalgam and magnesium. It has been established that this color reaction is due to the presence of the γ-benzopyrone nucleus. The details as used here have been set forth recently by Bryant (14). One milliliter of the alcoholic extract (representing 25–50 mg. of plant dry matter) was treated with 0.5 ml. hydrochloric acid (10%) and magnesium turnings. The color varied from pale to deep red depending upon the quantity of flavonoids. A score of one plus as used in this screening procedure indicated approximately 0.1% flavonoid present in the plant. This sensitivity was estimated by the addition of varying quantities of rutin to a known plant extract which initially gave a negative response to the test.

Alkaloids.—One milliliter of the water extract, prepared as described above, was acidified with 0.5 ml. of 1% hydrochloric acid and divided into two portions. To one was added 2 drops of Mayer's reagent (15, 16) [a potassium mercuric iodide solution described in (17)], and to the other was added 2 drops of silicotungstic acid (18)—12.0 Gm. of $4H_2O \cdot SiO_2 \cdot 12WO_3 \cdot 22H_2O$ in water sufficient to make 100 ml. If a precipitate was obtained either upon acidification or after addition of the reagents, a confirmatory test was performed in order to rule out interfering substances such as proteins.

About 1 ml. of water extract was made alkaline with 1% sodium hydroxide solution and extracted with an equal volume of chloroform. The chloroform extract was then extracted with an equal quantity of 1% hydrochloric acid and the latter extract divided into equal portions; one tested with Mayer's reagent, the other with silicotungstic acid solution. Scoring depended upon the quantity of precipitate obtained. On the basis of the silicotungstic acid test (using nicotine) a score of one plus indicated the presence of at least 0.01 to 0.1%, two plus indicated about 0.1 to 0.3%, and three plus over 0.3% alkaloids in the plant dry matter.

Tannins.—These were estimated by the response of the water extracts (prepared as described above) to gelatin-salt reagent (19) and confirmed by the black color produced with ferric chloride test solution (20, p. 939). Since precipitation with basic lead acetate occurred in every plant extract examined, whether or not tannin was found by other tests, this reagent was abandoned.

In the use of gelatin reagents a test was made with salt alone to guard against misinterpretation of results due to salting out effect which might be produced by nontannin constituents. Also, a separate test was made with 1% gelatin to aid in the evaluation of tannins since gelatin is less sensitive to them than gelatin-salt mixture and a positive test with gelatin alone indicates a larger amount of tannin present.

TABLE I.—OCCURRENCE OF THE VARIOUS SAPOGENINS IN THE SPECIES LISTED

Species	No. of Samples	Genin Content, M. F. B., % Min.	Max.
Chlorogenin			
Agave lecheguilla	1	...	0.1
Agave schottii	1	...	0.4
Agave sp.	2	tr	0.1
Chlorogalum pomeridianum	2	...	0.3
Yucca sp.	2	0.1	0.2
9-Dehydrohecogenin			
Agave deserti	1	...	tr
Agave huachucensis	1	...	0.1
Agave scabra	2	...	tr
Agave shawii	1	...	0.1
Agave sp.	1	...	0.2
9-Dehydromanogenin			
Agave goldmaniana	1	...	0.1
Agave nelsonii	1	...	tr
Agave shawii	2	tr	0.1
Diosgenin			
Agave sp.	1	...	0.2
Dioscorea composita	6	1.2	3.0
Dioscorea macrostachya	4	0.1	1.5
Dioscorea quaternata	2	0.3	0.4
Dioscorea sp.	7	0.1	2.0
Yucca filamentosa	1	...	0.3
Yucca sp.	1	...	0.1
Gitogenin			
Agave aurea	1	...	0.1
Agave filifera	1	...	0.2
Agave goldmaniana	1	...	tr
Agave nelsonii	1	...	0.1
Agave "pelon"	1	...	0.1
Agave schottii	1	...	0.8
Agave serrulata	1	...	0.1
Agave toumeyana	2	0.1	0.2
Agave sp.	8	0.1	0.3
Albuca sp.	2	tr	0.4
Chlorogalum pomeridiana	1	...	0.1
Yucca aloifolia	1	...	0.1
Yucca filamentosa	4	0.3	0.4
Yucca flacida	1	...	0.1
Yucca gloriosa	12	tr	0.2
Yucca thompsoniana	1	...	0.3
Yucca sp.	2	tr	0.1
Hecogenin			
Agave aurea	1	...	0.8
Agave chrysantha	1	...	0.5
Agave deserti	2	tr	0.1
Agave expansa	2	0.1	0.3
Agave goldmaniana	3	tr	0.2
Agave huachucensis	1	...	0.1
Agave nelsonii	2	...	0.1
Agave palmeri	1	...	0.5
Agave parryi	2	0.1	0.2
Agave cf. palonii	1	...	0.4
Agave scabra	2	0.1	0.2
Agave sebastiana	1	...	0.1
Agave serrulata	1	...	0.4
Agave shawii	2	0.1	0.2
Agave sobria	1	...	0.1
Agave toumeyana	4	0.1	0.3
Agave vexam	1	...	0.4
Agave sp.	14	tr	0.4
Yucca elata	1	...	0.1
Yucca gloriosa	3	tr	0.1
Yucca sp.	1	...	0.1

Species	No. of Samples	Genin Content, M. F. B., % Min.	Max.
Kammogenin			
Yucca filamentosa	3	...	0.1
Yucca gloriosa	1	...	0.1
Manogenin			
Agave aurea	1	...	0.2
Agave deserti	1	...	tr
Agave goldmaniana	4	tr	0.1
Agave mayoensis	1	...	tr
Agave nelsonii	1	...	tr
Agave cf. patonii	1	...	0.2
Agave serrulata	1	...	0.7
Agave shawii	2	tr	0.2
Agave toumeyana	2	0.1	0.2
Agave vexam	1	...	0.2
Agave sp.	1	...	0.2
Yucca filamentosa	1	...	0.1
Yucca gloriosa	5	tr	0.1
Sarsasapogenin			
Agave aff. attenuata	1	...	1.0
Yucca angustissima	1	...	0.3
Yucca baccata	3	0.2	0.5
Yucca elata	6	tr	0.7
Yucca gloriosa	1	...	0.2
Yucca intermedia	1	...	0.1
Yucca schidigera	2	0.4	0.5
Yucca standleyi	1	...	0.4
Yucca cf. thornberi	2	0.4	1.5
Yucca torreyi	1	...	0.5
Yucca valida	1	...	0.3
Yucca sp.	6	0.2	1.0
Yucca (leaf powder)	1	...	0.4
Samogenin			
Yucca carnerosana	1	...	0.3
Smilagenin			
Agave lecheguilla	5	0.1	1.0
Agave mayoensis	1	...	0.6
Agave aff. vilmoriniana	1	...	1.2
Agave sp.	8	0.2	0.9
Yucca aloifolia	1	...	0.1
Yucca carnerosana	1	...	0.1
Yucca sp.	1	...	0.1
Tigogenin			
Agave aurea	1	...	0.2
Agave goldmaniana	2	tr	0.1
Agave murpheyi	1	...	tr
Agave schottii	1	...	0.1
Agave serrulata	1	...	0.4
Agave shawii	1	...	tr
Agave sp.	6	tr	0.4
Albuca sp.	2	0.4	0.7
Chlorogalum pomeridiana	1	...	0.4
Yucca aloifolia	4	tr	0.2
Yucca elata	1	...	0.3
Yucca flacida	1	...	0.2
Yucca gloriosa	5	tr	0.2
Yucca sp.	4	tr	1.0
Yammogenin			
Dioscorea macrostachya	2	...	tr
Yuccagenin			
Agapanthus africanus	1	...	tr
Agapanthus umbellatus	2	0.1	0.2
Agave sp.	2	0.1	0.3
Nolina greenii	1	...	0.1
Yucca filamentosa	5	0.1	0.3
Yucca gloriosa	1	...	0.1
Yucca sp.	..	...	0.4

Since this Laboratory is interested in possible sources of tannins, C. W. Beebe made quantitative analyses (21) of some of the materials. Results obtained on several extracts are as follows:

Accession No.	Tannin, %	Screen Test Score
312	5.13	+
357	2.39	+
367	14.49	++
404	16.08	++
596	15.40	+++
727	18.04	+++
861	3.85	++
928	9.88[a]	++

[a] Analysis only approximate. Sample impossible to extract thoroughly. The scoring used in the screening procedure is in satisfactory agreement with the analytical values obtained.

Unsaturated Sterols.—Of the various color reactions for sterols (22) when they are treated with strong acids under dehydrating conditions, the Liebermann-Burchard and Salkowski tests were selected for this screening (23). Chloroform extracts prepared as described above were used and the scoring was made upon the basis of color intensities obtained. A score of one plus (+) indicated about 0.2% sterol content in the plant. This evaluation is based on the use of varying amounts of stigmasterol added to a plant extract which failed to produce the Liebermann-Burchard reaction. It should be pointed out, however, that the Liebermann-Burchard reaction is positive only in the presence of unsaturated sterols (24). Also, since the extracts tested had received no preliminary purification there is the possibility that other nonsteroidal unsaturated compounds (such as carotene and xanthophylls) may have interfered with this reaction (25). Where interference occurred, the color density appeared to be attained immediately. With spinasterol and similar sterols it did appear in this fashion. However, with most sterols and where interfering substances were not present, the color density was minimal immediately after the reaction began, then slowly increased, approaching a maximum in about fifteen to twenty minutes. In practically all of the samples tested in this screening work the color density was slow to develop and slow to reach a maximum.

Organic Acids and Phenols.—The term "organic acids" is used collectively in this work to apply only to water-soluble constituents of acid character. Five-milliliter portions of water extracts prepared as described under *Preparation of Extracts* were acidified with 2 ml. of 1% hydrochloric acid and extracted with 12 ml. of ether, the ether evaporated off spontaneously from a tared container, and the weight of air-dried residue recorded. The product has been termed "organic acids," but of course it would also contain nonvolatile phenols. Scoring of one-plus indicates less than 1% residue, two, 1–10%, and three, greater than 10%.

The qualitative scoring of phenols has been speculative. It has arbitrarily depended upon the tint and relative intensity of color produced with ferric chloride test reagent (20, p. 938). This test is of little value in the presence of tannins which produce a black color with ferric chloride. Absence

of organic acids above is indicative of the absence of nonvolatile phenols. The estimated value for phenols has therefore not been assigned higher than the figure given to organic acids.

RESULTS

A table has been prepared giving the data for each sample—its origin and identification, the results of the hemolysis tests, the kinds and amounts of steroidal sapogenins found, and the qualitative findings for flavonols, alkaloids, tannins, sterols, organic acids, and phenols. Because of space limitations this table cannot be given here. It has, however, been prepared in processed form as AIC–363 and may be obtained on request from the U. S. Department of Agriculture, Eastern Regional Research Laboratory, Bureau of Agricultural & Industrial Chemistry, Philadelphia 18, Pa.

Table I as included here is a rearrangement of the steroid data to show their occurrence by species.

A positive hemolysis test for saponin occurred in 60% of all species, but only one-fourth of these species contained steroidal saponins. There was a high incidence of steroidal sapogenins in certain genera: 47% in *Yucca*, 24% in *Agave* but, contrary to expectations, only 10% in *Dioscorea*. These steroids were found only in families of the Liliales—in the present list, Liliaceae, Amaryllidaceae, and Dioscoreaceae; two genera of Iridaceae had none. Of the 14 sapogenins found, diosgenin, gitogenin, hecogenin, sarsasapogenin, smilagenin, and tigogenin were in the greatest number of species. Kammogenin, samogenin, and yammogenin were rare and in small concentrations. Hecogenin was the predominant steroid in half the agaves; diosgenin in all of the yams; sarsasapogenin in 70% of the yuccas (26). *Y. filamentosa* was extremely varied, three different genins predominating in different samples.

The configuration at the ring A/B juncture at carbon 5 is with few exceptions a species characteristic. For example, many given *Agave* species form tigogenin, hecogenin, gitogenin, manogenin, and chlorogenin, singly or in a variety of combinations, all of which have the *trans* configuration at carbon 5. The corresponding *cis* form is never found in these species. *Agave lecheguilla* and a few other *Agave* species produce smilagenin with a *cis* structure at carbon 5, and the corresponding *trans* types are never found.

A similar situation is found in *Yucca*. Although a variety of sapogenins are produced by the various species, those which make the *cis* compounds never produce the *trans* and vice versa. In the *Dioscorea* the A/B juncture is a generic characteristic; only sapogenins unsaturated between carbons 5 and 6 are formed.

Regional specificity is particularly marked in *Yucca*. In agreement with Marker, *et al.* (3), we find that southwesterly yuccas growing in arid areas produce only sarsasapogenin. Those growing in the moist southeastern areas make a variety of sapogenins. In contrast to Marker, *et al.*, we find that smilagenin was not the predominant genin formed in the southeast. In most cases the sapogenins were C-5 *trans* compounds, tigogenin, and gitogenin being particularly common.

Flavonols were rarely found. A double-plus reaction was obtained only in four species of Liliaceae. A triple-plus was not obtained, although a sample of buckwheat leaves, *Fagopyrum esculentum* of about 4% rutin content, gave a triple-plus.

No alkaloids were found in *Agave*, but a triple-plus test was obtained in 14 other species of Amaryllidaceae, most of them from South Africa. A triple-plus was obtained in one species of Apocynaceae, in three of Dioscoreaceae, in two of Liliaceae, but not at all in *Yucca*. There is evidence that alkaloids do not occur in *Dioscoreas* native to the Western Hemisphere, but only in some Old World species (27). Table II lists the species in which alkaloids have been found for the first time.

TABLE II.—NEW ALKALOID-CONTAINING SPECIES

Amaryllidaceae
Ammocharis coranica (bulbs)
Ammocharis falcata (bulbs)
Brunsvigia rosea (whole)
Crinum buphanoides (bulbs)
Crinum cf. moorei (bulbs)
Haemanthus albiflos (bulbs)
Haemanthus coccineus (bulbs)
Haemanthus nelsonii (bulbs)
Leucojum aestivum (whole)
Narcissus cf. lazatta (bulbs, leaves)
Nerine angustifolia (bulbs)
Periphanes zeyheri (bulbs)
Vallota speciosa (bulbs)
Apocynaceae
Amsonia ciliata (whole)
Dioscoreaceae
Dioscorea dregeana (rhizome)
Liliaceae
Brodiaea uniflora (whole)
Polygonaceae
Rumex crispus (rhizomes)
Rumex hymenosepalus (rhizomes)

Tannin was absent from most families. In the Iridaceae it was found in four species of *Watsonia*. In the Liliaceae it was found in *Albuca*, *Nolina*, and *Smilax* (eight species). It was abundant in roots of *Rumex* (Polygonaceae), which is being considered as a commercial source.

Unsaturated sterols were generally frequent and abundant, mostly in leaves, but sometimes occurred in *Dioscorea* tubers.

A slight test for organic acids was obtained in practically all samples, as was to be expected. A two-plus test was obtained in only 32 samples, and these were mostly *Agave* and *Yucca*. One triple-plus was obtained in *Y. gloriosa*.

Even a slight test for phenols was obtained in only about 10% of the samples, and just one two-plus test, in *Y. gloriosa*.

Since these results for acids and for phenols seemed to lack significance or were fruitless, the tests for them were abandoned after the first 1,000 samples.

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The *in Vitro* Evaluation of the Antibacterial Activity of Sulfonamides*

By GEORGE M. NAIMARK and LESLY WHITE

The necessity for using bacteriological culture media containing a minimum of sulfonamide-inhibitors and capable of supporting satisfactory growth of the organism is emphasized. The other parameters significant in such bacteriostatic tests are reviewed. Thirty per cent sodium sulfacetamide solutions and ointments are shown to inhibit the growth of *M. pyogenes* var. *aureus* in a synthetic fluid medium.

THE STANDARD *in vitro* antibacterial tests cannot be casually applied to the evaluation of sulfonamides or sulfonamide-containing pharmaceuticals because the commonly used bacteriological culture media frequently contain sulfa-inhibitors which tend to mask the *in vitro* effects of these compounds. MacLeod and Mirick (1, 2) stated that the most important source of error in such bacteriostatic tests is the presence of sulfonamide-inhibitors in the usual bacteriological culture media and that unless inhibitor-free media are used, tests for the susceptibility of bacteria of any species to the sulfonamide drugs are unreliable and may be wholly misleading.

Known sulfonamide-inhibitors fall into two main classifications: substances of known composition (usually single compounds), and complex mixtures which have been incompletely characterized. The individual substances of known composition which inhibit the *in vitro* action of sulfonamides include *p*-aminobenzoic acid and related compounds, methionine, arginine, lysine, glutamic acid, glutamine, glycine, serine, allothreonine, guanine, xanthine, glucose, mercuric chloride, calcium chloride, and urethane (3). Incompletely characterized complex mixtures which have been found to be inhibitory to sulfonamide action include among others meat extracts and infusions, blood, plasma, serum, many proteins, peptones (protein hydrolysis products), necrotic tissue, and bacterial products (3).

In addition to the problem of sulfa-inhibitors, other parameters which significantly effect the results of any bacteriostatic test method include the following (4): (*a*) species and strain of test organism, (*b*) composition and *p*H of medium, (*c*) inoculum age, size, and diluting fluid, (*d*) concentrations and stability of drug solutions, (*e*) temperature and duration of incubation, and (*f*) criteria used to determine activity.

The importance of some of these variables is illustrated below. White indicated (5) that temperatures are critical accessory factors in the bacterial action of sulfonamides *in vitro* by showing that 100 times as much sulfanilamide was required for sterilization at 37° as was required at

* Received from Strong Cobb & Co., Inc., Cleveland 4, Ohio.
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TABLE I.—GROWTH OF M. PYOGENES VAR. AUREUS CULTURES

Test I, Sample	Hours at 37° C. 20 %	Hours at 37° C. 65 %
Inoculated Control—no sodium sulfacetamide	70.0	21.6
Solution No. 1 containing 30% sodium sulfacetamide	98.0	96.0
Solution No. 2 containing 30% sodium sulfacetamide	98.0	96.6

Test II, Sample	Hours at 37° C. 20 %	Hours at 37° C. 40 %
Inoculated control—no sodium sulfacetamide	28.1	25.0
Solution containing 30% sodium sulfacetamide	98.3	90.0
Ointment No. 1 containing 30% sodium sulfacetamide	97.3	95.0
Ointment No. 2 containing 30% sodium sulfacetamide	97.6	95.0
Ointment No. 3 containing 30% sodium sulfacetamide	98.0	96.0
Ointment No. 4 containing 30% sodium sulfacetamide	98.0	97.0

39°. White, *et al.*, (6) reported a thousandfold difference in sulfonamide activity with different test media. The literature is replete with similar observations which emphasize the necessity for the careful standardization of test conditions for the valid interpretation of data.

EXPERIMENTAL

The most common procedure utilized for *in vitro* sulfonamide tests involves the growth of bacteria in test tubes containing a liquid medium, which will satisfactorily support growth of the organism and which will be as inhibitor-free as possible. Since media based on natural products or their derivatives lead to irregular results, synthetic media composed of definite chemical entities are used to provide more uniform test results.

To test the effectiveness of sodium sulfacetamide and sodium sulfacetamide-containing products against *M. pyogenes* var. *aureus* (ATCC 6538P) the authors chose the medium originally described by Knight (7) and modified by Strauss (8). The medium was inoculated with 20,000 organisms and showed satisfactory growth in one day when incubated at 37°. Growth of the organism, as evidenced by increased turbidity, was usually evident in twelve hours. A sample of 0.1 Gm. ointment or solution was added directly to the tubes with no preliminary sample preparation. Growth was determined quantitatively by making turbidity measurements on a Coleman Model 11 Universal spectrophotometer (wave length 465 mμ, filter No. 4). Samples were read against uninoculated blanks.

The results in Table I are in terms of per cent transmission at various times of incubation.

The data clearly show the luxuriant growth of the organism in the absence of sodium sulfacetamide. The 30% sodium sulfacetamide solutions and ointments successfully inhibit the growth of *M. pyogenes* var. *aureus*.

SUMMARY AND CONCLUSIONS

1. Culture media essentially free of sulfonamide-inhibitors and capable of supporting satisfactory growth of the organism must be used for the valid *in vitro* evaluation of sulfonamide or sulfonamide-containing pharmaceuticals.

2. The culture medium described by Knight (7) and modified by Strauss (8) was satisfactory for the growth of *M. pyogenes* var. *aureus* although the sulfonamide-free controls did not attain full growth after the same period of incubation. The medium appears to be essentially inhibitor-free.

3. The growth of *M. pyogenes* var. *aureus* was inhibited under the conditions of this test by 30 per cent sodium sulfacetamide solutions and ointments.

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The Influence of Whole Body X-Irradiation on the Pharmacological Action of Certain Drugs*

By PATRICK F. BELCASTRO,† JOHN E. CHRISTIAN, and H. GEORGE DeKAY‡

A study was made to detect changes in the most prominent pharmacological action of certain drugs after they were administered to rats three days after whole body exposure to 540 roentgens of X-irradiation. There is some evidence that X-irradiated animals exhibit an altered response to epinephrine on blood pressure, atropine on gastric secretions, and ammonium chloride on diuretic action when compared with control or nonirradiated animals.

THE CLINICAL PICTURE after X-irradiation is evidenced by physiological and biochemical alterations in the processes essential for the life of the cell. The irradiated subject exhibits certain shocklike reactions and a series of gastrointestinal, hematological, and cardiovascular symptoms. These physiological changes have been attributed to a direct effect of ionizing radiations on the cell or to an indirect effect resulting when ionized molecules recombine to form new molecules which may have the ability to alter the functioning of cells. Some excellent reviews on the biological effects of ionizing radiations appear in the literature (1–4).

It is plausible that a pharmacological agent may not exert its normal effect on tissue in which the X-irradiated cell is a part, since drugs react with or influence specific cell constituents. The purpose of this project represents an attempt to detect any alteration in the most prominent pharmacological action of some commonly known drugs using rats which have been exposed to sublethal doses of X-rays. Changes in pharmacological activity may also reflect corresponding alterations in the rates of absorption, circulation, detoxification, and excretion of a drug through an indirect influence of ionizing radiations.

The majority of investigators have employed pharmacological or chemical agents to study the mechanism of the effects of ionizing radiations, in the numerous attempts to prevent their lethal effect and to alleviate the various symptoms induced by penetrating radiations. Very few papers appear in the literature which describe an evaluation of the degree of pharmacological action produced by drugs administered to X-irradiated animals. Rossi (5) exposed the rectus abdominus muscle of the frog to soft X-rays and found that the X-irradiated muscle recovered from atropine much earlier than the controls. Conard (6) studied the effect of X-irradiation on intestinal motility of the rat *in vivo* and employed various drugs to study the mechanism of action of X-rays on the intestine. Toyama (7) reported increased tone and motility with localized irradiation of the intestine and used nicotine and atropine to inhibit the response. Burn, *et al.* (8), investigated the responses of isolated loops of intestine taken from irradiated rats to acetylcholine and histamine and found increased responses to acetylcholine in the colon, but no similar changes to histamine were observed. Lovisatti (9) studied the influence of localized roentgen rays on the effect of adrenalin in the rabbit and found that X-irradiated animals are more resistant to lethal doses of adrenalin. Haley, *et al.* (10), studied the effects of acute whole body irradiation and reported a diminished response of the precapillary sphincters and terminal arterioles to epinephrine.

EXPERIMENTAL

White, adult, Sprague-Dawley rats of both sexes were subjected to total body X-irradiation in a single exposure, delivered at 200 kv., 15 ma., at a distance of 50 cm. from the tube using 0.5 mm. of copper and 1.0 mm. aluminum filters, at a rate of approximately 54 roentgens per minute. The beam was directed downward and exposures were given to the animals through a plastic box 15.5 x 18 cm. and 4.4 cm. high and divided into five perforated compartments to allow adequate ventilation. Five unanesthetized animals weighing from 120 to 150 Gm. were irradiated at one time. The area of the irradiation field was 20 x 20 cm. All animals used in this study received 540 roentgens of X-rays, originating from a General Electric Maximar therapy unit.[1]

After irradiation ten animals were placed in a cage and allowed access to Purina Laboratory Chow and water during a postirradiation period of three days. An equal number of control or nonirradiated animals were set aside under the same conditions

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† Fellow of the American Foundation for Pharmaceutical Education.
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[1] The authors are indebted to Dr. Donald C. McClelland, radiologist at the Home Hospital in Lafayette, Indiana, for the generous use of his X-ray machine.

and at the same time as treated animals. The loss of weight occurring each day after X-irradiation was observed and was employed in this project as a means of establishing a criterion for estimating the severity of physiological damage induced by the X-irradiation. Figure 1 illustrates the mean loss in weight occurring in 100 irradiated animals during the postirradiation period. An analysis of variance indicated a linear relationship between the quantity of weight loss *versus* time. The deviations from a linear regression were not significant ($F=0.19$). Loss in weight occurring after three days is approximately the same as that reported in a previous publication (11).

On the third day after irradiation various drugs were administered to both control and irradiated animals to produce the following pharmacological actions: analgesia, local anesthesia, central depression, central stimulation, sympathomimetic action, sympatholytic action followed by epinephrine, diuresis, and parasympathetic depression on gastric secretions. The details of the methods of evaluation and the results are given under the separate class headings.

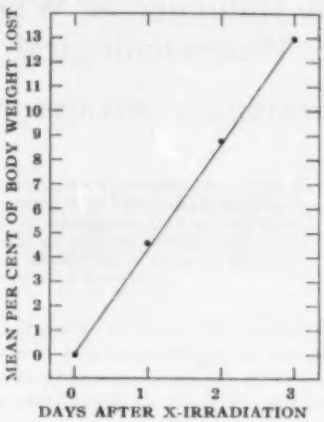


Fig. 1.—Per cent loss in weight after whole body X irradiation—540 Roentgens. 100 animals.

RESULTS

Analgesic Activity.—Morphine sulfate, 10 mg./Kg., was injected subcutaneously. The degree of analgesia was observed every fifteen minutes for a period of three hours by determining the rat's reaction time in seconds to a heat stimulus applied to a small area of the tail. The method is essentially that described by Davies, *et al.* (12). The mean onset and duration of analgesic action for ten control and ten irradiated animals is plotted in Fig. 2 which illustrates the close proximity between the mean responses of both groups. A statistical analysis of the data showed no significant difference between the means of the control and irradiated rats.

Central Depressive Activity.—Sodium pentobarbital, 40 mg./Kg., was injected intraperitoneally. The duration of a state of "sleep" was determined from the time the animals lost their righting reflex to the period when they resumed it. The method is described by Fitch and Tatum (13). The mean duration of hypnosis for 32 X-irradiated animals was 145 ±6.60 (S. E.) minutes, and 137 ±7.9 (S. E.) minutes for 40 control animals. The means are statistically insignificant ($t = 0.75$).

Central Stimulant Action.—The convulsive dose of Metrazol was found to be 55 mg./Kg. for the Sprague Dawley rat and was injected subcutaneously to ten control and ten X-irradiated animals. The time of onset, duration, and strength of the convulsions were qualitatively observed in an attempt to detect possible differences in susceptibility to the drug. There were no obvious differences in this respect, between the control and X-irradiated animals.

Local Anesthetic Action.—The method of Rose (14) was used in which 0.1 cc. of a 1% procaine hydrochloride solution is injected intradermally and the resulting "wheal" is stimulated every two minutes by means of two electrodes connected to a Harvard Inductorium. The mean duration of local anesthesia for ten control animals was 121 ±12 (S. E.) minutes as compared to 98 ±8 (S. E.) minutes for the irradiated animals. Differences between

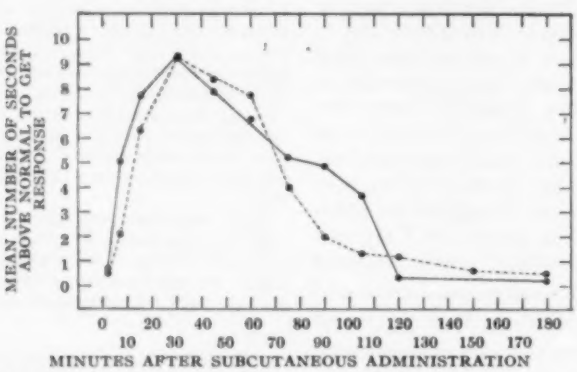
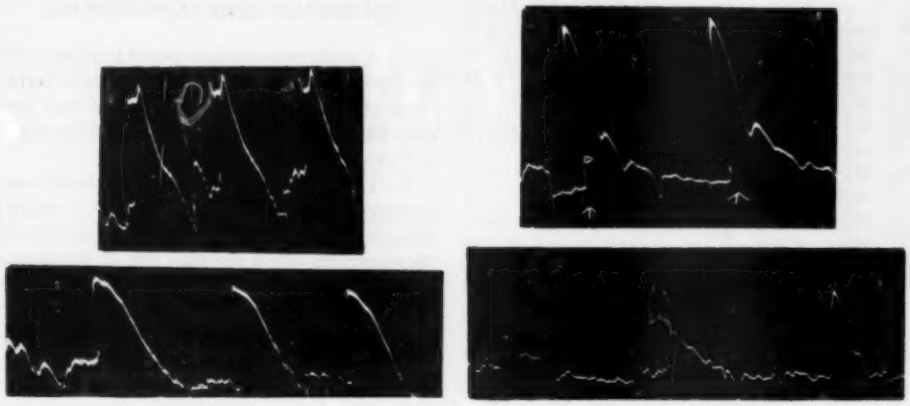


Fig. 2.—Mean duration of morphine analgesia in control and X-irradiated animals. ⊙ - - - - - ⊙ Controls; ⊙ ——— ⊙, Irradiated.



Control animals X-irradiated animals

Fig. 3.—Typical blood pressure responses to epinephrine 0.5 μg.

the means are statistically insignificant ($t = 1.434$) at the 5% confidence level.

The failure to observe differences in the pharmacological action of the above drugs is evidence of the resisting nature of nerve tissue to this level of X-irradiation as has been observed by Snider (15) and others. The indirect effects of X-rays apparently had no influence on the dominant pharmacological action of the above drugs.

Sympathomimetic Action.—The effect of epinephrine, 0.5 μg./animal, ephedrine sulfate 2.0 mg./Kg., Tuamine 10 mg./Kg., and epinephrine 0.5 μg. after dibenamine 25 mg./Kg., on blood pressure was determined by cannulation of the right carotid artery of atropinized and anesthetized (urethan, 1 Gm./Kg.) rats. All drugs were administered through the femoral vein. Interpretation of the kymograph record illustrated in Fig. 3 suggests that X-irradiated rats exhibit a tendency toward a decreased response in blood pressure after administration of epinephrine. The duration of the peak blood pressure rise after epinephrine is not as long in X-irradiated animals as that observed in control animals. The illustrated responses are typical of those obtained in twenty irradiated animals.

Ephedrine sulfate and Tuamine both produced a typical prolonged rise in blood pressure in both control and X-irradiated animals. The epinephrine blood pressure reversal after Dibenamine was observed to be identical in both irradiated and control animals. There were no apparent differences between irradiated animals and controls in regard to initial response or duration of action of these three pharmacological agents.

The reduced sensitivity of the X-irradiated rat to epinephrine may be explained on the basis of an increased metabolism which has been observed in the irradiated animal (16) and which would probably tend to increase the rate of oxidation of epinephrine. Since ephedrine and Tuamine exhibited their usual prolonged rise in blood pressure, this possibly suggests that the reduced sensitivity to epinephrine may be attributed to an indirect effect of ionizing radiations rather than to a direct effect on the arterioles. Haley (10) applied epinephrine directly to the terminal arterioles and observed a decreased sensitivity which he attributed to the presence of a vasodepressor substance in the blood stream of the irradiated animal which causes a vascular paralysis and therefore a reduced sensitivity to the vasoconstrictor action of epinephrine.

Atropine Action on Gastric Secretions.—The effect of 1 mg./Kg. of atropine sulfate on gastric secretions was studied according to the technique of Morris, *et al.* (17), in which the pyloric sphincter of fasted animals is ligated under anesthesia. The animals were sacrificed by chloroforming, a 1-cc. aliquot of the stomach contents was removed and titrated with 0.01 normal sodium hydroxide solution using Topfer's reagent and phenolphthalein as indicators for the determination of the free and total acidity, respectively. Results are expressed in Table I.

TABLE I.—GASTRIC ACIDITY BEFORE AND AFTER ATROPINE ADMINISTRATION TO CONTROL AND IRRADIATED ANIMALS

No. of Rats	Treatment	Mean Free[a] Acidity	Fisher t	Mean Total[a] Acidity	Fisher t
13	None	31 ± 4[b]		58 ± 4[b]	
16	Irradiated (540 r.)	40 ± 3	2.08	71 ± 2	2.93
22	Atropine (1 mg./Kg.)	8 ± 2	2.34	37 ± 3	
16	Irradiated-atropine	16 ± 3		39 ± 2	0.47

[a] Refers to the number of milliliters of 0.1 N NaOH necessary to neutralize 100 cc. of stomach contents.
[b] Refers to standard error of the arithmetic mean calculated as follows:

$$\text{S. E.} = \frac{\sigma}{\sqrt{N}}$$

An examination of Table I shows that X-irradiation alone (no drug) causes a slight increase in free and total acidity ($t_{0.05} = 2.06$). The data also suggest that 1 mg./Kg. of atropine sulfate will not lower free acidity to the same extent observed in control aniamls ($t_{0.05} = 2.03$). Atropine appears to have no influence on the total acidity of irradiated animals. Although the above data are not significant, they denote a trend toward a decreased action

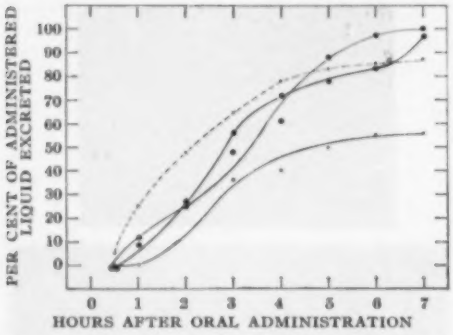


Fig. 4.—The rate of urine excretion after urea administration to control and X-irradiated animals. ⊙ Urea, 3 days after x-irradiation; ⊖, urea, 5 days after x-irradiation; x saline solution 25 cc./Kg.; ● urea, controls.

of atropine on the free acidity of irradiated animals. This phenomenon may be due to a direct stimulation of parietal cells to produce more HCl, to a lowering of the alkaline reserve of the blood, or to circulatory disturbances caused by the X-irradiation.

Diuretic activity.—The method of Lipschitz, *et al.* (18), was employed for the determination of diuretic activity of urea, 1.5 Gm./Kg., and ammonium chloride 0.3 Gm/Kg. The rate of urine excretion during seven hours after the administration of urea to irradiated and control animals is portrayed in Fig. 4. There was no apparent significant difference between the mean rate of urine excretion in irradiated and control animals.

Ammonium chloride shows a marked decreased ability to produce diuresis in irradiated animals. This effect is evidently temporary since, after post-irradiation periods of six, twelve, and fifteen days, surviving animals exhibited a progressive rise in diuretic acitivity. Results are summarized in Table II.

This temporary reduction of ammonium chloride diuretic activity may be attributed to a dehydrated state characteristic in irradiated animals, to possible liver damage, or to a modified blood mineral balance. Bloom (19) reported no histological damage to the convoluted tubules after 600 roentgen irradiation.

TABLE II.—AMMONIUM CHLORIDE DIURESIS X-IRRADIATED ANIMALS

No. of Rats	Treatment	Days after Irradiation	Per Cent Excretion[b] after 8 Hours
5	Saline—25 cc./Kg.	...	73
30	NH_4Cl—0.3 Gm./Kg.[a]	...	139
10	NH_4Cl—irradiated	3	78
10	NH_4Cl—irradiated	6	114
6	NH_4Cl—irradiated	12	143
5	NH_4Cl—irradiated	15	158

[a] The drug was dissolved in 25 cc./Kg. of physiological saline solution.

[b] % Excretion = $\frac{\text{Total volume of urine excreted for } n \text{, number of animals}}{\text{Volume of saline solution of drug fed to the same number of animals}}$

SUMMARY AND CONCLUSIONS

1. A study was made to detect changes in the most prominent pharmacological action of certain drugs after they were administered to rats three days after whole body exposures to 540 roentgens of X-irradiation.

2. There is a diminished response to the vasoconstrictor action of epinephrine in X-irradiated animals as indicated by a decreased duration of the maximum blood pressure rise in the cannulated rat.

3. Ammonium chloride diuretic activity is temporarily decreased when administered to rats on the third day after X-irradiation.

4. There is some evidence that atropine sulfate, 1 mg./Kg., does not lower the free gastric acidity in irradiated animals to the same extent observed in control animals.

5. X-irradiation has no apparent effect on the major pharmacological action of morphine sulfate, sodium pentobarbital, Metrazol, procaine, ephedrine, Tuamine, epinephrine after Dibenamine, atropine on total acidity, and urea on diuretic activity, when studied in the intact rat employing the methods described.

6. It is not to be inferred from the results observed in this study that the same events will necessarily occur in other animal species or with other levels of X-irradiation under different experimental conditions.

7. There is a need for studying the biological effects of X-rays on the pharmacological action of drugs at a cellular level in order to elucidate this problem further.

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Composition of Gum Turpentine of Pines*

XVIII. A Report on *Pinus pungens, P. glabra,* and *P. teocote*

By N. T. MIROV, P. M. ILOFF, Jr., and L. B. GORDON†

Pinus pungens Lamb. (table-mountain pine) is a tree which in a forest occasionally reaches 60 feet in height, with a trunk two to three feet in diameter; when open-grown, it is rather scrubby—20 to 30 feet high, with a short, thick trunk. Its cones are two to three and one-half inches long, roundish, and armed with hooked, curved spines. *Pinus pungens* grows on dry gravelly slopes and ridges of the Appalachian Mountains from southern Pennsylvania to North Carolina, eastern and middle Tennessee, and Georgia. A sample of oleoresin was collected from trees of this species in Buncombe County, N. C., at an elevation of 2,400 feet. Unfortunately, some of the liquid part of the sample was lost in transit, and the yield of turpentine was rather low—only 14.5%. The turpentine was separated from the oleoresin *in vacuo*, so that at the end of the distillation the temperature was 170° and pressure was 1 mm. The turpentine possessed the following characteristics: d_4^{23}, 0.8564; n_D^{23}, 1.4682; $[\alpha]_D$, −23.7.

A batch of 200 Gm. of the turpentine was distilled in a 90-cm. Todd column equipped with a heated jacket and a stillhead permitting a 10:1 reflux ratio. Results of the distillation are shown in Table I.

Fraction 2 was tested for presence of α-pinene. Pinene nitrosochloride[1] recrystallized three times from chloroform by means of methanol had a melting point of 106.5°.

Fractions 10 and 11 combined were tested for presence of β-pinene[1] by oxidizing the oil with 24 Gm. of potassium permanganate and 5 Gm. of sodium hydroxide in 600 cc. of water. Water was then evaporated in an open beaker to 200 cc. Abundant precipitate of sodium nopinate was formed upon cooling the solution. Nopinic acid was prepared by dissolving sodium nopinate in dilute sulfuric acid and was extracted by shaking the solution with ether. After three recrystallizations from benzene, nopinic acid melted at 126–127°.

Limonene was identified in Fraction 17 by preparing a tetrabromide[1] possessing a melting point of 117–118°. The pot residue (6.5 Gm.) boiling above 176° was redistilled in a 10-cc. flask. More than 3 Gm. distilled at 175–176°; the remaining oil became dark and apparently partly polymerized.

Judging by the distillation behavior, *Pinus pungens* gum turpentine consisted of about 70% *l*-α-pinene; 20% *l*-β-pinene and 8–9% *l*-limonene. The polymerized residue amounted only to about 1% of the weight of the oil.

Pinus glabra Walt. is the spruce pine of the southeastern United States. In South Carolina it is called king's tree; in Florida, poor pine, which shows the inconsistency of common names. Spruce pine attains its largest size—up to 120 feet in height—in northwestern Florida. It grows as far west as southeastern Louisiana.

A sample of oleoresin was obtained by John K. Gross, U. S. Forest Service, from five trees growing in the DeSoto National Forest, Perry County, Miss. Oleoresin, 3,500 Gm., was distilled under reduced pressure. Toward the end of the distillation, the temperature of the oleoresin was 150°; the pressure was 0.05 mm. The yield of turpentine was 22.3%.

The turpentine possessed these characteristics: d_4^{23}, 0.8599; n_D^{23}, 1.4735; $[\alpha]_D$, −4.65°.

A batch of 500 Gm. was subjected to fractional distillation in a Todd column 90 cm. long and 25 mm. inside diameter. The reflux ratio was 10:1. The results are given in Table I.

Fraction 2 was examined for α-pinene. Pinene nitrosochloride melted at 107°; β-pinene was identified in fraction 10 by preparing nopinic acid, which, after three recrystallizations from benzine, melted at 125°.

Fraction 18 (6 cc.) was brominated in 1:1 ether-ethanol mixture. An abundant precipitate was formed when 3.5 cc. of bromine was added. The melting point of the tetrabromide after several recrystallizations from ethyl acetate was 106°.

Fraction 21 was tested for a sesquiterpene. Dry HCl gas was bubbled through a solution of the oil in absolute ether. Crystals were formed after the treated solution was kept under refrigeration for two weeks. These crystals, however, melted at room temperature. By suction filtration while cold, some white crystals, which melted at 48–49°, were isolated.

Pinus teocote Schlecht et Cham. is a widely distributed, variable Mexican pine, growing at elevations ranging from 4,500 to 10,000 feet. It occurs from the Northern States of Durango, Coahuilla, and Nuevo Leon to Guatemala. It has a casual resemblance to *Pinus leiophylla* and the natives often call both by the same name, "pino chino." Sometimes it is called "pino rosillo." A sample of oleoresin of this pine was collected under the supervision of Forest Engineer, Cenobio E. Blanco—an expert on Mexican pines—at El Salto, Durango, at an elevation a little over 8,000 feet.

A batch of 914 Gm. of the oleoresin was distilled at reduced pressure, so that at the end of distillation, the temperature in the flask reached 203° and the pressure was 0.8 mm. The yield of turpentine was 24.2%.

The crude turpentine had the following physical characteristics: d_4^{23}, 0.8578; $n_D^{21.8}$, 1.4669; $[\alpha]_D^{23}$, +7.6°.

* Received July 14, 1953, from the Institute of Forest Genetics, California Forest and Range Experiment Station, maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.

† The work reported in this paper was aided through a grant from the Rockefeller Foundation.

[1] For methods used see previous articles of this series in THIS JOURNAL.

TABLE I.—FRACTIONAL DISTILLATION OF TURPENTINES OF PINUS PUNGENS, P. GLABRA, AND P. TEOCOTE

Fractions	Boiling Range, °C.	Distillate, %	Density, d^{23}_4	Index of Refraction n^{20}_D	Optical Rotation $[\alpha]^{23}_D$, Degrees
		Pinus pungens (200 Gm. Used)			
1	150–155	0.9	...	1.4630	−10.0
2	155–156	42.8	0.8549	1.4663	−10.0
3	156–157	17.5	...	1.4672	−10.7
4	157–158	3.0	...	1.4700	−11.2
5	158–159	4.7	...	1.4700	−12.0
6	159–160	2.4	...	1.4715	−13.7
7	160–161	0.9	...	1.4727	−13.7
8	161–162	1.4	...	1.4740	−16.0
9	162–163	2.0	...	1.4753	−17.0
10	163–164	4.0	0.8614	1.4770	−18.0
11	164–165	3.2	0.8612	1.4770	−20.0
12	165–166.5	3.2	...	1.4773	−24.2
13	166.5–168	2.1	...	1.4770	−30.0
14	168–169	2.0	...	1.4760	−36.0
15	169–171	2.2	...	1.4767	−44.0
16	171–173	1.3	...	1.4759	−54.3
17	173–176	3.0	0.8423	1.4750	−70.5
Residue and losses	...	3.4	...	...	...
		Pinus glabra (500 Gm. Used)			
1	155–157	1.4	0.8556	1.4678	+16.5
2	157–158	20.5	0.8563	1.4683	+16.5
3	158–159	10.5	0.8577	1.4694	+12.5
4	159–160	6.4	0.8586	1.4710	+ 8.3
5	160–161	5.1	0.8584	1.4720	+ 4.4
6	161–162	5.4	0.8584	1.4732	+ 0.7
7	162–163	3.6	0.8584	1.4743	− 3.3
8	163–164	6.1	0.8607	1.4754	− 7.4
9	164–165	11.9	0.8617	1.4772	−13.3
10	165–166	14.3	0.8617	1.4784	−19.0
11	166–167	4.2	0.8614	1.4785	−23.3
12	167–168	2.0	0.8593	1.4782	−28.4
13	168–169	1.3	0.8580	1.4779	−34.0
14	169–171	1.1	0.8548	1.4770	−40.7
15	171–173	0.6	0.8506	1.4767	−50.0
16	173–175	1.6	0.8447	1.4759	−62.7
Continuation of distillation at 10 mm. pressure:					
17	60–62	0.4	0.8433	1.4755	−67.7
18	62–63	1.2	0.8443	1.4764	−66.3
19	63–65	0.6	0.8497	1.4776	...
20	65–75	0.1	0.8574	1.4801	−60.0
21	75–90	0.2	0.8623	1.4949	−25.0
22	90–95	0.1	...	1.5000	...
23	95–125	0.1	...	1.5007	...
Residue and losses	...	1.3	...	...	...
		Pinus teocote (203 Gm. Used)			
1	151–154.8	3.7	0.855	1.4657	+ 7.5
2	154.8–156	82.6	0.855	1.4661	+ 7.6
3	156–157	1.6	0.855	1.4673	+ 5.8
4	157–161	2.5	0.854	1.4711	+ 0.8
Continuation of distillation at 8 mm. pressure. Fraction 8 at 1 mm.:					
5	58–76	1.6	0.855	1.4783	+ 2.0
6	76–86	0.4	0.872	1.4805	+ 4.0
7	86–90	1.2	0.905	1.4890	+ 8.2
8	80	1.5	0.930	1.5007	+30.6
Residue and losses	...	4.9	...	...	...

A batch of 203 Gm. of the turpentine was fractionated in a 90-cm. 12-mm. inside diameter Todd column packed with glass helices. A reflux ratio of 12:1 was maintained. After a distillate equal to 90.4% of the charge had been received at atmospheric pressure, the residue in the pot (13.1 Gm.) was distilled at reduced pressure through a 3-in. Vigreux column at reduced pressure, using a 4:1 reflux ratio. The results of the fractionation are shown in Table I.

The first two fractions had constants close to those of α-pinene. During the collection of fraction 2 there was almost no variation in physical properties and the majority of the fraction boiled over a 0.2° range. α-Pinene was identified in fraction 2 by preparation of *dl*-α-pinene nitrosochloride, which

melted at 103–104° after two recrystallizations from chloroform by addition of cold methanol. A mixed melting point determination with authentic *dl*-α-pinene nitrosochloride showed no melting point depression. A nitrolpiperidide, m. p. 120°, was also prepared.

Fraction 8 had the properties of a sesquiterpene. From 1.4 Gm. of fraction 8, 0.4 Gm. of crude sesquiterpene hydrochloride was prepared. The sesquiterpene hydrochloride melted at 58–59° after two recrystallizations from glacial acetic acid. There was no depression in melting point upon admixture with a sample of hydrochloride from *d*-longifolene derived from *P. ponderosa* from the Black Hills, South Dakota.

To sum up, *Pinus pungens* turpentine consisted of *dl*- and *l*-α-pinene, 70%; *l*-β-pinene 20%; *l*-limonene 8–9%. *Pinus glabra* turpentine contained *dl*- and *d*-α-pinene, 45%; *l*-β-pinene, 45%; *l*-limonene, 8%; and possibly a small amount of sesquiterpene containing hydrochloride with very low melting point. *P. teocote* from Durango, Mexico, yielded turpentine consisting of 92% *dl*- and *d*-α-pinene, and 3% of sesquiterpene, the major constituent of which is *d*-longifolene.

Nitroalkyl Ethyl Carbonates*

By A. KNEVEL† and C. E. MILLER‡

A laboratory method has been developed for the preparation of nitroalkyl ethyl carbonates. Four new mixed carbonates were synthesized in which the nitroalkyl group varied from butyl to heptyl.

THE PROPERTIES of aromatic nitro compounds have been known for many years. However, our knowledge of aliphatic nitro derivatives is considerably less. The physiological activity of certain nitro alkyl sulfonates has been reported (1). Because of the activity shown by this structure, it seemed worth-while to prepare a series of nitro alkyl carbonates for pharmacological testing. The information obtained from this testing would be a contribution to our knowledge of the relationship between chemical structure and pharmacological action.

A survey of the literature revealed that alkyl carbonates had been known since 1873 when Lieben (2) reported the preparation of di-*n*-butyl carbonate. Chattaway (3) extended the study of carbonates by reacting phosgene with aliphatic alcohols. No mention was found in the literatue pertaining to nitroalkyl carbonates. It is the purpose of this paper to report our method of preparing mixed nitroalkyl carbonates by the reaction of ethyl chloroformate and nitroalkyl alcohols together in the presence of a suitable catalyst.

EXPERIMENTAL

Procedure.—The procedure developed for the preparation of the carbonates listed in Table I is outlined in detail. One-half mole of ethyl chloroformate and 0.5 mole of the nitroalcohol were placed in a 1-L. three-neck flask fitted with a mechanical stirrer, dropping funnel, and a thermometer. Twenty-two grams (0.55 mole) of sodium hydroxide previously dissolved in 200 ml. of distilled water and cooled to room temperature was added dropwise to the rapidly stirred mixture while the reaction temperature was maintained at 20–25° by external cooling. Agitation was continued for an hour after addition of the aqueous alkali. Sufficient distilled water was added to the flask to dissolve the sodium chloride, the mixture transferred to a separatory funnel, and the salt solution drawn off. The water-immiscible liquid was washed free of chlorides and dried over anhydrous sodium sulfate. Vacuum distillation yielded unreacted starting material, the desired product, and a high boiling residue. The mixed carbonates were light yellow in color, had a fruity odor, did not react with α-naphthyl isocyanate, and gave a positive qualitative test for a nitro group. Table I gives the physical and chemical constants of the mixed ethyl carbonates prepared from 2-nitrobutanol-1,[1] 2-nitro-2-methylpropanol-1,[1] 4-nitro-2-methylhexanol-3, and 3-nitroheptanol-4. The latter two nitroalcohols were prepared by the method of Vanderbilt (4).

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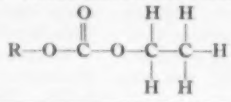
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.

† Abstracted from a thesis presented to the Graduate Council of North Dakota Agricultural College by A. Knevel in partial fulfillment of the requirements for the degree of Master of Science. Acknowledgment is given to the Cancer Society, Inc., for financial assistance during this investigation.

‡ Professor of Pharmaceutical Chemistry, School of Pharmacy, North Dakota Agricultural College, Fargo, N. D.

[1] Generously supplied by Commercial Solvents Corporation.

TABLE I.—NITROALKYL ETHYL CARBONATES



No.	R	Yield, %	B. P., °C./mm.	$(n)_D^{21}$	Empirical Formula	Analysis, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	2-nitrobutyl	31	96–97/5	1.4235	$C_7H_{13}NO_5$	43.91	43.57	6.83	6.93
2	2-nitro-2-methylpropyl	37	93–95/5	1.4285	$C_7H_{13}NO_5$	43.91	43.38	6.83	6.86
3	4-nitro-2-methylhexyl-3	36	91–92/6	1.4420	$C_{10}H_{19}NO_5$	51.48	51.62	8.20	8.13
4	3-nitroheptyl-4	39	101–102/6	1.4434	$C_{10}H_{19}NO_5$	51.48	51.62	8.20	8.12

SUMMARY

1. A method for the sodium hydroxide catalyzed reaction between ethyl chloroformate and aliphatic nitroalcohols has been developed.

2. The following new mixed nitroalkyl ethyl carbonates have been prepared: 2-nitrobutyl ethyl carbonate; 2-nitro-2-methylpropyl ethyl carbonate; 3-(4-nitro-2-methyl)-hexyl ethyl carbonate, and 4-(3-nitro)-heptyl ethyl carbonate.

3. These compounds are being tested pharmacologically and will be reported elsewhere.

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A Pharmacological Study of a Crystalline Glycoside of *Caulophyllum thalictroides**

By H. C. FERGUSON† and L. D. EDWARDS‡

Pharmacological studies demonstrate that the outstanding action of a crystalline glycoside isolated from *Caulophyllum thalictroides* is that of a smooth muscle stimulant.

THE RHIZOMES and roots of *Caulophyllum thalictroides* (Blue Cohosh) were used by the early Indians to facilitate childbirth, and were considered a very valuable remedial agent among eclectic and homeopathic practitioners (1).

The plant has been the subject of considerable chemical investigation since 1863 when Mayer reported that the alcoholic tincture, left to spontaneous evaporation, deposited a white granular substance giving alkaloidal tests (2). Lloyd, in 1887, verified Mayer's findings and named the alkaloid caulophylline and confirmed Ebert's finding of a glycoside (1, 3).

Power and Salway continued the work and determined the chemical composition of these and other principles (4). They found the alkaloid (N-methylcystisine), two crystalline glycosides, an enzyme, fixed oil, volatile oil, and citrullol.

Davy and Chu (5), in 1927, attempted to isolate the crystalline glycosides found by Power and Salway, but they recovered an alcohol-soluble glycoside which was not crystallizable and differed from that described by Power and Salway. They concluded that the only method by which they could obtain a crystalline glycoside was by moistening the drug with water to allow enzyme action prior to extraction.

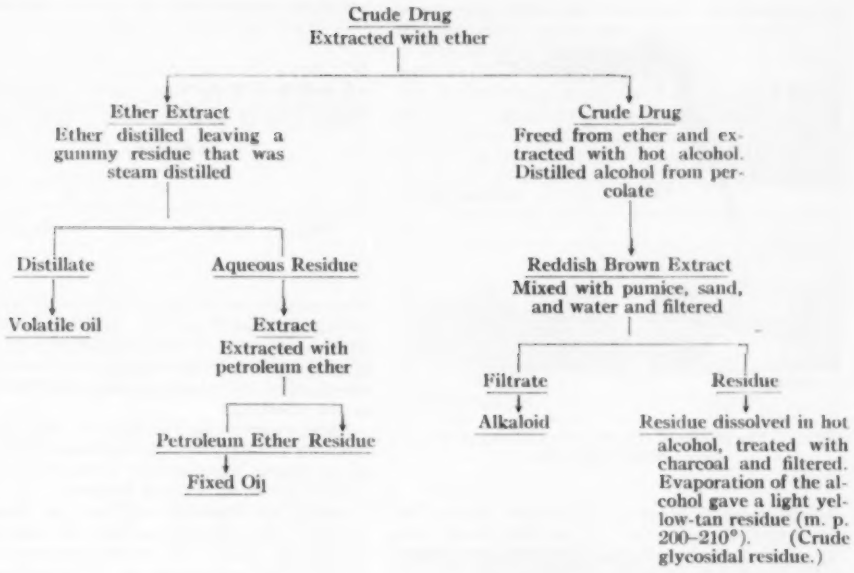
In 1943 Scott and Chen (6) determined the pharmacological activity of the alkaloid. Very little work has been done on the pharmacological activity of the glycosides (4). Such a study of one of these crystalline glycosides constitutes the basis of this report.

* Received August 21, 1953, from the School of Pharmacy, Purdue University, Lafayette, Ind.

Abstract of a dissertation submitted to the Graduate School of Purdue University by H. C. Ferguson in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Fellow of the American Foundation for Pharmaceutical Education. At present, Assistant Professor of Pharmacology, University of New Mexico, College of Pharmacy.

‡ Professor of Pharmacology, Purdue University, School of Pharmacy.


Crude Drug
Extracted with ether
Ether Extract
Ether distilled leaving a gummy residue that was steam distilled
Crude Drug
Freed from ether and extracted with hot alcohol. Distilled alcohol from percolate
Distillate
Aqueous Residue
Reddish Brown Extract
Mixed with pumice, sand, and water and filtered
Volatile oil
Extract
Extracted with petroleum ether
Filtrate
Residue
Alkaloid
Petroleum Ether Residue
Residue dissolved in hot alcohol, treated with charcoal and filtered. Evaporation of the alcohol gave a light yellow-tan residue (m. p. 200–210°). (Crude glycosidal residue.)
Fixed Oil

EXPERIMENTAL

Isolation of the Crystalline Glycoside.—The whole rhizome and roots were ground and passed through a No. 20 sieve. The above flow sheet outlines the extraction procedure followed:

The alcohol-soluble residue gave a negative test with Fehling's solution prior to acid hydrolysis and a positive test after acid hydrolysis. It is insoluble in most solvents with the exception of sodium hydroxide (*p*H 11.8), which effects a solution which may then be diluted a number of times. The residue showed a definite oxytocic acitivity on the rat uterus, therefore methods of purification were undertaken.

If the glycosidal residue is dissolved in U. S. P. alcohol, a brown solution is produced. The failure of the glycoside to separate or crystallize from this alcoholic solution is probably due to impurities which block crystallization. However, if the crude residue is first treated with cold anhydrous alcohol, most of the impurities are dissolved. The alcohol is then filtered and the residue is dissolved in boiling anhydrous alcohol. Upon cooling, the glycoside crystallizes slowly in acicular tufts. These have a brown tinge, but upon recrystallization from alcohol the coloring is removed leaving a white product which melts at 255–256°. The crude drug yields approximately 0.1% of these crystals.

The crystalline glycoside (hereafter referred to as *G*) was also insoluble in most solvents except sodium hydroxide (*p*H 11.8), and propylene glycol heated to 50°. The sodium hydroxide solution was employed for pharmacological testing. After *G* is solubilized by sodium hydroxide, the resulting solution can be diluted as much as ten times with saline or distilled water without evidence of precipitation. This seems to indicate that a sodium salt is formed which is soluble in water to a much greater extent than the original glycoside.

In all pharmacological tests equivalent doses of the above solvents were used as controls.

The crystalline product occurs as pure white, feathery crystals; it yields a reducing sugar on acid hydrolysis, has a slight acrid taste, is odorless but irritating to the nasal mucosa, its solubility in water is very slight, a red color is produced when it is warmed with concentrated sulfuric acid, and a pink color changing to yellow upon warming with concentrated nitric acid.

PHARMACOLOGY OF THE CRYSTALLINE GLYCOSIDE

Action on Uterus.—Doses of 0.25–0.5 mg./70 ml. of aerated Locke-Ringer's solution maintained at 38° produced on the rat uterine horn, in early estrus, an increase in the degree and rate of contraction. Larger doses gave an immediate increase in tone and rate of contraction, but decreased the degree of contraction. When washed with Locke-Ringer's the horn returned to normal. A dose of 1 mg./70 ml. bath effected a contraction which was sustained for approximately ninety minutes. Similar results were obtained with the rabbit and guinea pig uterus. With rat uterine strips, in early pregnancy, a dose of 0.5 mg./70 ml. bath produced a primary stimulation followed by a period of inhibition. After these alternate periods had continued for about one hour, a second dose (0.5 mg./70 ml. bath) resulted in an immediate increase in tone (Fig. 1). With the rat uterus *in situ* (urethan, 1.2 Gm./Kg.) 5 mg./Kg., intravenously, produced an increase in the degree of contraction and a slight increase in the rate of contraction. Larger doses, 8–10 mg./Kg., effected a rapid and pro-

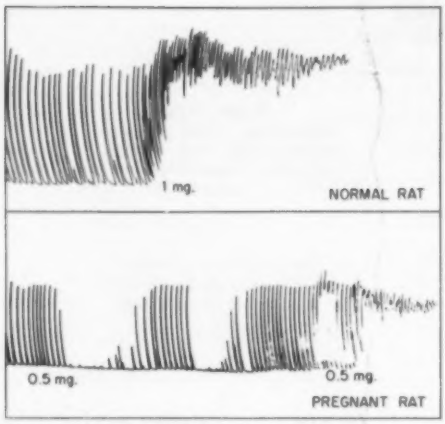


Fig. 1.—The effects of *G* on the normal and pregnant uterus of the rat *in vitro*.

nounced increase in tonus with a slight decrease in the height of contraction and an increase in rate (Fig. 2). Here, the increase in tonus was maintained for approximately forty-five minutes.

Figure 3 shows the results on the *in vivo* rat uterus. The method employed is as follows:[1]

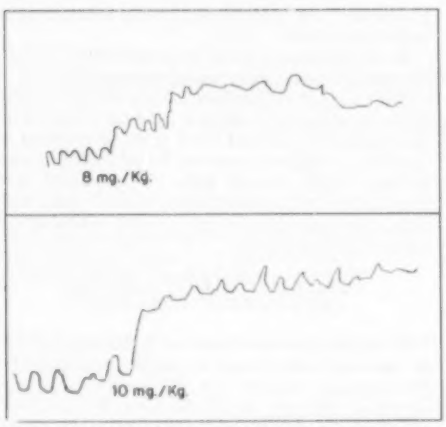


Fig. 2.—The effects of *G* on the rat's uterus *in situ*

Albino rats, 175–250 Gm., in late estrus, were anesthetized by subcutaneous injection of 1 cc./Kg. of a 25% urethan solution containing 30 mg./cc. of pentobarbital sodium. The rat was then secured to an operating board, ventral side up, and the vagina filled with warm Locke-Ringer's solution. The flanged end of a medicine dropper was then inserted into the vagina and tied in place. A *T*-tube was used to connect the vaginal cannula to a sensitive tambour. Pressure within this closed system was altered by the injection or removal of Locke-Ringer's solution by means of a syringe. The pressure necessary for the onset of peristaltic type uterine movements varied per rat. In such a preparation intravenous doses of the glycoside of 8–10 mg./Kg. produced tonus changes similar to but much reduced as compared to the tonus changes noted in the above *in situ* experiment.

[1] Suggested by Dr. T. S. Miya, School of Pharmacy, Purdue University.

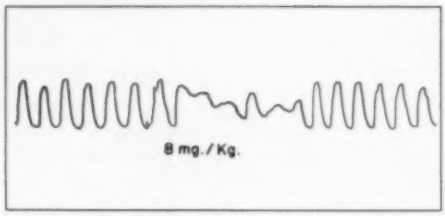


Fig. 3.—Effects of the rat's uterus *in vivo*.

Eight assays using the U. S. P. XII method for posterior pituitary injection indicate that 1 mg. of the glycoside possesses the activity of 0.00065± 0.000041 (S. E. M.) units of Pituitrin.

Action on Intestine.—*Effects on Longitudinal Muscles.*—The Magnus method (7) was employed to show the action of *G* on the intestine of the rat, mouse, rabbit, and guinea pig. The intestinal strips were suspended in an aerated bath of Sollmann-Rademaker's solution (8) maintained at 38°.

Doses of 0.1–1.5 mg./70 cc. bath were used. Normal strips and strips treated with atropine (1:100,000) showed a spasmogenic effect with a decrease in the degree of contraction. The guinea pig's intestine showed an initial decrease in tonus before the predominant increase (Fig. 4).

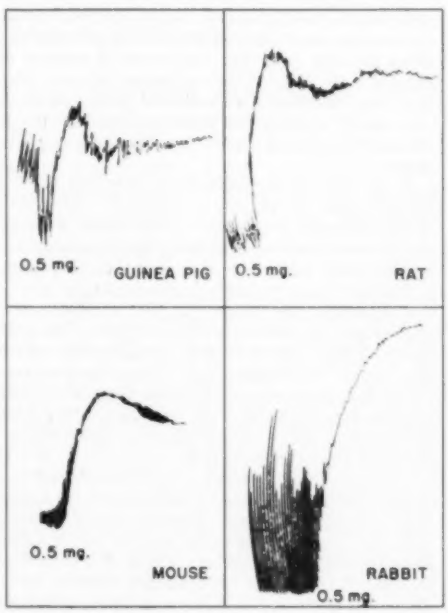


Fig. 4.—Effects on intestinal contractions involving primarily the longitudinal muscles.

Peristalsis Modifications.—The method employed was that developed and used by Picchioni and Edwards (9).

In the case of the guinea pig, doses of 1–2 mg./250 cc. bath produced a primary stimulation followed by depression that eventually resulted in abolition of all peristalsis (Fig. 5).

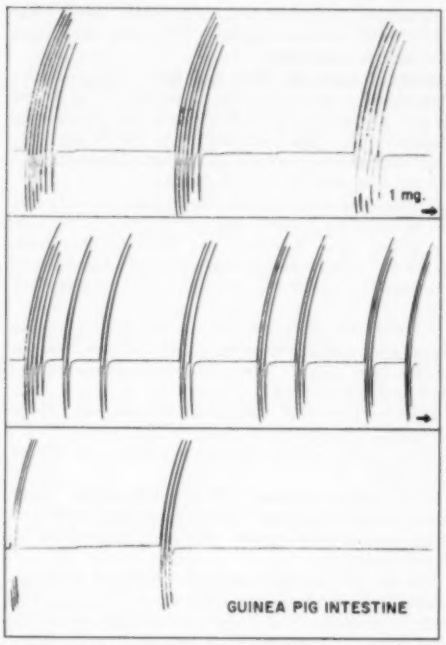


Fig. 5.—Effects on peristaltic movements *in vitro.*

Much smaller doses were required to produce the same effect on the rat's intestine.

Blood Pressure Studies.—Twelve intact rats, twelve pithed rats prepared according to the method described by Shipley and Tilden (10) with the exception that a respirator as designed by Edwards and Miya was used (11), and one dog showed a very transient drop in blood pressure followed by a slight rise above normal. The results did not warrant further study.

Perfusion of the Turtle and Frog Heart in Situ.—Perfusion of 12 turtle hearts with doses of 0.3–0.6 mg., and 12 frog hearts with doses of 0.05–0.14 mg. showed a positive tonotropic effect with a slight bradycardia. Doses larger than those designated above produced cardiac irregularities that led to a partial heart block culminating in systolic arrest. A series of small doses eventually produced the toxic effects shown by one large dose, thus indicating a cumulative action (Fig. 6).

Effects on Mammalian Heart.—*Rat Heart Perfusion.*—Twelve rat hearts were perfused by the Langendorff technique (12). The heart was perfused at a pressure of 40 mm. of Hg with oxygenated Locke-Ringer's solution prepared with double distilled water. The temperature of the perfusate was regulated so that it was 38° when it entered the heart. A pin was inserted into the ventricular apex and the thread passed over a pulley and connected to a heart lever to record contractions. Coronary flow was taken simultaneously.

A dose of 10 μg. contained in a volume of 0.1 cc. produced a positive inotropic effect followed by a more lasting negative inotropic effect. A slight tachycardia was observed. The mean reduction in coronary flow was 25.6%±1.36 (S. E. M.) in twelve trials.

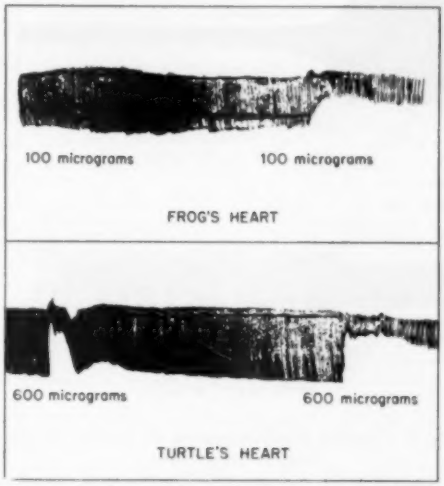


Fig. 6.—Typical results produced by the perfusion of the cold blooded heart *in situ.*

Pithed Rat's Heart.—The method employed was that used by Ponich (13). Albino rats, 200–250 Gm., were anesthetized with an intraperitoneal injection of urethan, 1.2 Gm./Kg. The trachea was cannulated and the animal pithed according to the method previously noted. The heart was then exposed. The xiphisternum was reflected anteriorly. The heart was freed by incising the pericardium and a hook was attached to the ventricular apex. A glass tube, 45 mm. long and 16 mm. in diameter was used to protect the heart from mechanical disturbances and also served as a moist chamber. The glass chamber was placed around the heart at a 30–40° angle to the rat leaving the lobes of the lungs free to move about the outside of the tube. The heart was moistened at frequent intervals with a few drops of Locke–Ringer's solution. The animal was allowed a thirty minute adjustment period before injections were made into the iliac vein.

Doses of 2 mg./Kg. produced a positive inotropism accompanied by a slight tachycardia lasting for fifty minutes. Doses greater than 4 mg./Kg. produced a great increase in contractility culminating in diastolic stoppage (Fig. 7).

Action on Arterial Spirals of Cattle and Hogs.—The arterial spiral preparation described by Lewis and Koessler (14) was used. Beef and hog carotid arteries were obtained from a local abattoir and were used as soon as possible after the death of the animal. Spirals refrigerated for twenty-four hours were still suitable for use.

Doses of 2–10 mg./70 cc. produced a contraction of the carotid spiral proportional to the dose.

Irritation Studies.—*The Determination of Local Irritation by Subcutaneous Injection into the Dorsal Surface of the Rabbit's Ear.*—The procedure and method of scoring used by Kuna and Cuchie were employed (15).

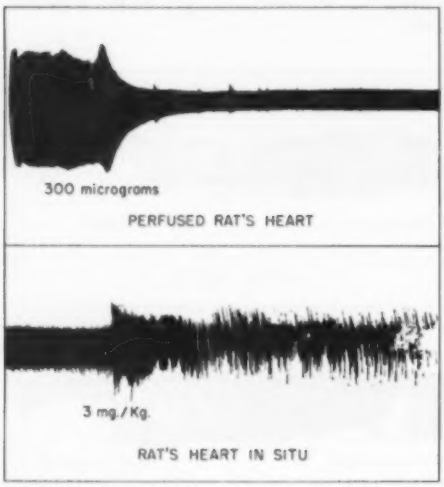


Fig. 7.—Typical results produced on the rat's heart *in vitro* and *in situ*.

Subcutaneous injections of three concentrations of *G* in propylene glycol were made. The dilutions used were 0.5%, 1%, and 2%. Injections were made into the ears of 6 rabbits in a volume of 0.05 cc. Each ear was injected at two sites, that is an injection of the solvent and one of the dilutions of the drug. Taking into consideration both the area and the depth of the lesion produced, the degree of irritation was scored on the extent of erythema, edema, and necrosis. The numerical values were recorded at the end of twenty-four and forty-eight hours and the sum of the numerical values for the three concentrations was used as an indication of the degree of irritation.

The score for *G* was 97 and for propylene glycol 63. Although irritation was shown by the solvent alone, *G* plus the solvent produced a much greater degree of irritation. It may be concluded that *G* in propylene glycol is too irritant for subcutaneous injection in the concentrations used in these tests.

Rabbit's Cornea.—A 0.5% solution in propylene glycol, when instilled in the rabbit's eye for one minute, produced corneal opacity to the extent that it was almost impossible to observe the pupil size. Pronounced redness of the conjunctiva, edema to the extent that the lids were half closed, and conjunctival discharge which kept large areas around the eye moist for several hours were also observed. This marked inflammation lasted for twenty-four hours and then the eye was washed with a weak solution of sodium bicarbonate. At the end of forty-eight hours after instillation of the drug, the inflammation was greatly reduced and a normal condition was reached in seventy-two hours. A slight inflammation was produced with propylene glycol, but in no matter did it compare with the irritation caused by *G*.

Hemolytic Action on Red Blood Cells.—In concentrations of 1:10,000 and greater, *G* shows a lytic action on the red blood cells of the rabbit and the rat. The hemolytic activity was not altered by *p*H, the ions contained in Locke-Ringer's solution, or blood serum.

Attempts to prevent hemolysis by the use of vitamin C (16), sodium salicylate (17), and cholesterol (18) were unsuccessful.

Acute Toxicity in Mice and Rats.—White Swiss mice of either sex, 20–40 Gm., were fasted for twelve hours. Six groups with 6 animals per group were employed and doses ranging from 10–15 mg./Kg. were administered intravenously in a concentration of 0.5% in sodium hydroxide (*p*H 11.8).

Albino rats of either sex, 65–90 Gm., were fasted for twenty-four hours. Six groups with 7 animals per group were employed and doses ranging from 18–23 mg./Kg. were administered intravenously in concentration of 0.4% in sodium hydroxide (*p*H 11.8).

All animals were kept at 26–29° in individual cages and mortalities were taken at the end of twenty-four hours. The LD_{50} was calculated according to De Beer (19).

Mice—LD_{50} 11.8 mg./Kg. Limits of Error 93–107%.

Rats—LD_{50} 20.3 mg./Kg. Limits of Error 92.5–108%.

Small doses produced a depression while larger doses caused increased activity, ataxia, and terminal clonic convulsions. Death appeared to be due to asphyxia.

Effects of Prolonged Administration of *G* in the Albino Rat.—Albino rats received 25% subcutaneously of the LD_{50} of *G*, in propylene glycol, daily for sixty days. No symptoms of toxicity or gross pathology of the heart, liver, spleen, intestine, kidney, or uterus were observed. Microscopic examination showed a slight edema of the epithelium of the uriniferous tubules in the kidney, and a thickening in the arterial walls in the spleen.[3]

Hydrolysis of the Glycoside.—Upon acid hydrolysis rhombohedral crystals were formed (m. p. 310–312°), that were insoluble in most solvents except sodium hydroxide (*p*H 11.8) containing 50% alcohol. The aglycone produced the same uterine action as did the glycoside.

DISCUSSION

Following the extraction procedure first used by Davy and Chu (5), an alcohol-soluble residue similar to that described by them was obtained. Our experiments have shown that it is possible to obtain a crystalline glycoside (*G*) from the alcohol-soluble residue. *G* shows the behavior of the class of substances known as saponins and its solubility in sodium hydroxide indicates an acid saponin that forms a water-soluble salt. The compound appears to be identical to leontin isolated by Lloyd (1) and caulosaponin isolated by Power and Salway (4). *G*, on

[3] This examination was made by Dr. L. P. Doyle of the Purdue Agricultural Experiment Station.

acid hydrolysis, yields a crystalline product corresponding to the aglycone obtained from the hydrolysis of caulosaponin.

SUMMARY

1. A crystalline glycoside, showing a definite oxytocic action, has been isolated from *Caulophyllum thalictroides*.

2. It constricts the coronary vessels of the rat's heart and carotid arteries of cattle and hogs, and has a spasmogenic effect on the isolated intestine of the rat, guinea pig, mouse, and rabbit.

3. The glycoside shows a toxic action on cardiac muscle, probably due to the action on the coronary vessels.

4. Subcutaneous injection into the dorsal surface of the rabbit's ear, and instillation into the rabbit's eye shows the glycoside to be very irritating.

5. Acute and "repeated-dose" toxicity data are presented

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WHO MAKES IT?

The Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Illinois, seeks information on sources of supply of the following chemicals:

Sodium pentathionate
Boron tribromide
Acenaphthalene
1,7-Dimethylxanthine
Tetramethylethylene oxide
Di-*tert.*-butylsilicon dichloride
Anthracene-1-carboxylic acid
3,4-Dimethylpyridine
9,10-Diaminoanthracene
beta-Aminoisobutyric acid
6-Methylindole
1,4-Dibromo-2-butyne
3,5-Dihydroxycinnamic acid
Pyrazine-2,3-dicarboxylic acid
4-Methyltryptophane
Glycyrrhetinic acid
Amygdalin
delta-Tocopherol
Ornithuric acid
Protoanemonin
Ruthenium nitrate
Di-*tert.*-butyl magnesium
Rubidium sulfide
Indazole
Fluoroiodomethane
2,4,5-Trimethyl-1,4-hexadiene
1,2,5-Trihydroxy-2-methyl-4-pentanone
2-Methylol-1,3-dioxolane
N-Methyl-6(1)-pyridone-3-carboxamide
2,1,3,4-Tetrazole-5-carboxylic acid
cis-Diphenylethylene oxide
4-Methoxyproline
1-methylxanthine
Capsaicin
Cinchol
Kynurenine
l-Limonene
Gymnemic acid

The Ultraviolet Spectrophotometric Determination of Official Phenobarbital Preparations*

By LELAND N. MATTSON†

An ultraviolet spectrophotometric method of assay for phenobarbital in official phenobarbital preparations is described. Procedures are presented utilizing the optical density of phenobarbital solutions in a borate buffer at *p*H 9.5 to obtain assay results. The results of 21 assays are reported with satisfactory agreement with known or labeled values. The assay method is shown to be applicable to phenobarbital tablets containing stearates. A change in the absorption characteristics of phenobarbital allowed to stand in 2 per cent sodium hydroxide is reported.

THE OFFICIAL METHODS for the assay of phenobarbital preparations in the United States Pharmacopeia and National Formulary are time consuming and, in the case of phenobarbital tablets containing stearates, require special treatment of the sample before proceeding with the extraction of phenobarbital. Several workers (1–5) have reported ultraviolet absorption assays for barbiturates in blood and tissue, and Goldbaum (6) has recently described the assay and differentiation of various barbiturates found in biological materials in microquantities. In 1940 Elvidge (7) presented a method for the assay of phenobarbital tablets which made use of the optical density of a solution of phenobarbital in normal sodium hydroxide at 256 mμ and later Stuckey (8) determined absorption spectra for a number of barbiturates in various media including sodium hydroxide solution. The well-known instability of barbiturates at high *p*H levels has been pointed out by various investigators including Husa and Jatul (9). However it was found that a solution of phenobarbital in a buffer having a *p*H of 9.5 exhibited the same optical density after standing twenty-four hours at room temperature as when prepared and that the value for $E_{1cm.}^{1\%}$ at 240 mμ was 459 at this *p*H. Others (1, 6) have found a peak absorption at 240 mμ for phenobarbital in solutions having a *p*H of 9 to 11.

After consideration of these observations it was decided to attempt to establish an ultraviolet procedure applicable to the assay of official and unofficial phenobarbital preparations in order to effect a considerable saving in time.

* Received June 8, 1953, from the Chemical Laboratories, Cole Chemical Company, St. Louis 8, Mo.
† The assistance of Dr. Arthur J. Zimmer, Associate Professor of Pharmaceutical Chemistry, St. Louis College of Pharmacy, in the preparation of this manuscript is gratefully acknowledged.

EXPERIMENTAL

Optical density was measured with a Beckman DU spectrophotometer equipped with ultraviolet accessories.

Reagents.—Alcohol, U. S. P.

Boric Acid and Potassium Chloride, 0.1 *M*. Dissolve 6.185 Gm. of boric acid and 7.455 Gm. of potassium chloride in water and dilute to 1,000 cc. with water.

Sodium Hydroxide, 0.1 *N* prepared carbonate free and standardized volumetrically.

A buffer solution having a *p*H of 9.5 is conveniently prepared by adding 34.43 cc. of 0.1 *N* sodium hydroxide to 50 cc. of 0.1 *M* boric acid potassium chloride solution and diluting to 100 cc. with water.

Procedure for Phenobarbital Tablets U. S. P.—Transfer a finely ground sample containing about 25 mg. of phenobarbital to a 250-cc. volumetric flask and dilute to volume with buffer solution. Mix thoroughly and allow to stand with occasional shaking for ten minutes. Filter through Whatman No. 41 paper, discarding the first 25 cc. of filtrate and transfer a 10-cc. aliquot to a 100-cc. volumetric flask. Dilute to volume with buffer solution, mix and measure optical density at 240 mμ using buffer solution as a reference. The results of a series of tablet assays by the described method and the same samples assayed by the U. S. P. extraction method are compared in Table I. Also in Table I is shown the results of the ultraviolet spectrophotometric assay of a tablet mixture containing no stearates and the result of the same tablet mixture with a stearate added in the usual proportion as lubrication and assayed by the ultraviolet spectrophotometric method.

TABLE I.—COMPARISON OF ULTRAVIOLET SPECTROPHOTOMETRIC AND U. S. P. ASSAYS OF PHENOBARBITAL TABLETS

Sample	Labeled Amount of Phenobarbital, Gr.	U. S. P. Assay, Found, Gr.	Ultraviolet Spectrophotometric Method, Found, Gr.
A	0.50	0.515	0.491
B	0.50	0.510	0.511
C	0.50	0.503	0.490
D	0.50	0.494	0.515
E	0.25	0.250	0.251
F[a]	0.25		0.255
G[b]	0.25		0.255

[a] Tablet mix containing no stearate.
[b] Same mix as (a) with stearate added.

Phenobarbital Elixir U. S. P.—Preliminary experiments established that of the several materials included in the elixir base only amaranth gave any interference at 240 mμ. This interference is very slight and a satisfactory correction is made by use of a blank reference containing elixir base without phenobarbital and treated in the same manner as the sample.

Procedure.—Transfer 5 cc. of elixir to a 100-cc. volumetric flask and dilute to volume with buffer. Mix thoroughly and transfer a 5-cc. aliquot of this solution to a 100-cc. volumetric flask and dilute to volume with buffer. Mix thoroughly and read optical density at 240 mμ using as a reference an elixir base without phenobarbital treated in the same manner as the sample. Table II shows the results obtained on U. S. P. elixirs and also elixirs labeled "Not U. S. P." containing 3.84 Gm. of phenobarbital per L. The elixirs labeled "Not U. S. P." were found to contain interfering substances. In order to eliminate these interferences a modification of the above procedure was necessary. A 5-cc. aliquot was transferred to a 100-cc. volumetric flask, diluted to volume with alcohol and mixed. A 5-cc. aliquot was evaporated to dryness in a 250-cc. beaker on a water bath and the residue dissolved in exactly 100 cc. of buffer solution. The optical density was then measured using buffer solution as reference.

TABLE II.—RESULTS OF ASSAY OF PHENOBARBITAL IN PHENOBARBITAL ELIXIR U. S. P.

Sample	Phenobarbital, Gm./L.	Phenobarbital Recovered by Spectrophotometric Assay, Gm./L.
A	4.0	3.96
B	4.0	3.95
C	4.0	3.93
D	4.0	3.93
E	4.0	4.09
F[a]	3.84	3.88
G[b]	3.84	3.86

[a] and [b] Labeled "Not U. S. P."

Ephedrine Sulfate and Phenobarbital Capsules.—Two procedures for the assay of phenobarbital in ephedrine sulfate and phenobarbital capsules were investigated, the first using the combined aqueous alkaline extract from a Hilty and Wilson (10) type extraction after the removal of ephedrine and dilution to appropriate concentration with buffer before measuring the optical density; and the second method using a direct dilution of the sample in buffer and measurement of the optical density with correction being made for the ephedrine sulfate absorption calculated on the basis of the ephedrine sulfate found on a separate sample determined by the method of Hilty and Wilson (10).

Procedure No. 1.—Transfer a sample containing approximately 100 mg. of phenobarbital to a separator, dissolve in 10 cc. of 2% sodium hydroxide solution and extract with five 25-cc. portions of ether. Combine ether extracts and wash with three 15-cc. portions of water, washing the water through 25 cc. of ether and adding to the alkaline aqueous portion. The ether portion is reserved for the determination of ephedrine sulfate by extraction with standardized sulfuric acid. Transfer the combined aqueous alkaline solution to a 500-cc. volumetric flask and dilute to volume with buffer solution. Mix thoroughly and transfer a 5-cc. aliquot to a 100-cc. volumetric flask and dilute to volume with buffer solution. Mix thoroughly and read optical density at 240 mμ.

Procedure No. 2.—Transfer a sample containing about 100 mg. of ephedrine sulfate to a separator and determine the ephedrine sulfate as outlined in Procedure No. 1, discarding the aqueous alkaline extract. Transfer a second sample containing approximately 25 mg. of phenobarbital to a 250-cc. volumetric flask and dilute to volume with buffer solution. Mix and transfer a 10-cc. aliquot to a 100-cc. volumetric flask and dilute to volume with buffer solution. Mix and measure optical density at 240 mμ. Correct the reading for ephedrine sulfate interference, using as a basis for calculation the percentage of ephedrine sulfate found by extraction and $E^{1\%}_{1\,cm.}$ 240 mμ = 3.32 for ephedrine sulfate at *p*H 9.5.

The corrected optical density is calculated by use of the following formula:

$ODC = OD - (e \times 3.32)$, where
ODC = corrected optical density
OD = observed optical density
e = Gm. of ephedrine sulfate in final 100-cc dilution
3.32 = $E^{1\%}_{1\,cm.}$ 240 mμ for ephedrine sulfate at *p*H 9.5.

Results obtained in the assays of ephedrine sulfate and phenobarbital capsules are summarized in Table III.

TABLE III.—RESULTS OF PHENOBARBITAL ASSAYS ON EPHEDRINE AND PHENOBARBITAL CAPSULES BY ULTRAVIOLET SPECTROPHOTOMETRIC METHOD

Sample	Phenobarbital Mg.	Phenobarbital Recovered Mg.
	Procedure No. 1	
A	100	96.30
B	100	96.80
C	100	96.67
	Procedure No. 2	
A	100	100.4
B	100	100.2
C	25	25.00
D	25	25.15

Discussion.—No difficulties were encountered in the assay of phenobarbital tablets or elixir, and it was found that an assay could be completed in about thirty minutes amounting to a considerable saving in time over the extraction procedure which requires two hours for drying time, exclusive of the time required for extraction and evaporation of solvent or possible removal of stearates.

Since the values found for phenobarbital using procedure No. 1 in the assay for phenobarbital in ephedrine sulfate and phenobarbital capsules were somewhat lower than expected although well within the tolerance range allowed by the National Formulary (11) and it had been shown that phenobarbital solutions were unstable at high *p*H (9, 12) it was thought that some deterioration of the sample might have occurred even though the extraction procedure allowed contact with a solution of 2% sodium hydroxide for little more than an hour. To determine the possibility and extent of this deterioration, one sample of ephedrine sulfate and phenobarbital was allowed to stand in 2% sodium hydroxide solution for two hours before determination by procedure No 1 and a second sample was allowed to stand fifteen hours in 2% sodium hydroxide before extracting the ephedrine and determining the phenobarbital as described in Procedure No. 1. Two hours, delay in

starting the extraction procedure resulted in a loss of 6.7% of phenobarbital present and a delay of fifteen hours showed a loss of 39.14% of the known amount of phenobarbital. In view of these facts it would seem that Procedure No. 2 would be the better of the two methods described.

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The Antibacterial Activity of an Extract Obtained from *Ascophyllum nodosum**

By DIAMANTE D. VACCA† and ROBERT A. WALSH‡

Monthly extracts obtained from *Ascophyllum nodosum* were tested for antibacterial activity *in vitro* against 11 organisms received from the American Type Culture Committee. The F. D. A. agar plate method of testing was used. The extracts were treated chemically to determine their composition. A seasonal variation in the activity of the extracts is discussed.

SINCE THE DISCOVERY of penicillin most workers have studied the molds and actinomycetes extensively as sources of antibacterial agents of vegetable origin. However in 1951, Pratt and his co-workers investigated the algae of the Pacific Coast and found that certain species yielded extracts which exhibited antibacterial activity (1).

As a result of Pratt's work, it was decided to investigate some of the algae along the Atlantic Coast. At first, two species, *Ascophyllum nodosum* (Linnaeus) and *Enteromorpha compressa* (Linnaeus), were collected. The alcoholic extracts were screened against laboratory cultures of *Escherichia coli* and *Micrococcus pyogenes* var. *aureus* for activity. Only the extract obtained from *Ascophyllum nodosum* gave promising results, and it was decided to investigate this species more thoroughly.

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† An abstract of a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by Diamante D. Vacca, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
‡ Formerly Assistant Professor of Biology and Pharmacology, Massachusetts College of Pharmacy. Presently: Director of Research, The E. L. Patch Company, Stoneham, Mass.

EXPERIMENTAL PROCEDURE

Collection.—Nahant, Mass., was chosen as the site of collection because of the accessibility of its rocky coast. *Ascophyllum nodosum* adheres quite firmly to the rocks and must be collected at low tide. At times when the weather was favorable, the monthly samples were collected in 5-gallon wide-mouth jars containing sea water and were transported to the Bacteriology Laboratory of the Massachusetts College of Pharmacy.

Extraction.—The plants were prepared for extraction by draining them of excess sea water and cutting them into small segments. At first the whole plant was extracted, but the whole plant did not give as great a yield of extract as did the plant cut into small segments. The following solvents were tried and evaluated: alcohol, U. S. P., chloroform, U. S. P., and ether anhydrous and purified. Alcohol proved to be the solvent of choice because extracts obtained with the other agents exhibited no activity. The sample was extracted for forty-eight hours and the solvent evaporated spontaneously. The extracts were then heated for four hours at 55° to insure complete removal of the solvent.

Extract.—The extract obtained was a dark, brownish-green, solid mass with a characteristic odor and with the consistency of molasses upon being heated. It was very slightly soluble in alcohol and practically insoluble in water. The antibacterial activity of the extract was not affected when the extract was stored at room temperature for four months. The flame test showed the presence of sodium and the absence of potassium. Nitrogen, sulfur, and iron were present; the halogens were absent. There was only a 39% loss upon ignition.

Testing.—The method of testing was the F. D. A. Agar Plate Method (2).

The organisms used at first were laboratory cultures of *E. coli*, *Bacillus subtilis*, *Micrococcus pyogenes* var. *aureus*, *M. pyogenes* var. *albus*, and *Sal-*

TABLE I.—ACTIVITY OF MONTHLY EXTRACTS FROM JULY, 1952, THROUGH JANUARY, 1953

Organisms Used	Radius of Inhibition Zones from Monthly Extracts Recorded in Centimeters							Average Inhibition
	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	
E. coli	2.0	1.3	1.5	1.5	0.8	0.9	0.7	1.24
Ps. aeruginosa	1.4	1.3	1.5	1.7	1.3	1.2	1.1	1.35
M. pyogenes var. *albus*	1.2	1.0	1.8	1.2	0.8	0.8	0.8	1.08
B. subtilis	0	0	0	0	0	0	0	0
A. aerogenes	1.3	1.2	1.4	1.5	0.9	0.8	0.7	1.11
M. pyogenes var. *aureus*	1.5	2.2	2.3	1.9	0.9	0.8	0.6	1.45
Br. melitensis	1.4	1.0	1.2	0.5	0.6	0.7	0.7	0.87
S. typhosa	2.0	2.0	3.2	1.5	1.0	1.1	0.9	1.81
Kl. pneumoniae	1.5	1.6	1.0	1.7	1.2	1.2	1.0	1.31
Pr. vulgaris	1.3	1.5	1.0	1.4	1.0	1.1	1.0	1.18
S. pyogenes	1.0	1.2	0.8	0.8	0.4	0.3	0.4	0.68

monella typhosa. After incubation the plates were examined for inhibition of growth. The radius of the zones of inhibition in each instance was measured and recorded. During the latter part of the investigation, transfers were made from the zones of inhibition to determine whether the action was bactericidal or bacteriostatic. The extracts obtained on and after the July, 1952, collection were tested against cultures of known strains obtained from the American Type Culture Committee. These test organisms were as follows: *E. coli* 26, *Ps. aeruginosa* 9027, *M. pyogenes* var. *albus* 151, *B. subtilis* 6051, *Aerobacter aerogenes* 8308, *M. pyogenes* var. *aureus* F. D. A. 209, *Brucella melitensis* 8360, *S. typhosa* F. D. A. Hopkins 6539, *Klebsiella pneumoniae* Type *A* 8045, *Proteus vulgaris* 8427, and *Streptococcus pyogenes* 4543.

Precautions to avoid contamination were taken while the extracts were being tested. The incubators were maintained at a temperature of 37°, and aseptic technique was used throughout the entire procedure. Gram-stained preparations were prepared from the daily cultures and from both the plates and the broth tubes after incubation, to confirm the absence of any contaminants.

RESULTS AND CONCLUSIONS

The monthly alcoholic extracts obtained from *Ascophyllum nodosum* showed *in vitro* antibacterial activity against ten of the 11 organisms tested. The extracts were active against some pathogenic and nonpathogenic organisms, of both Gram-positive and Gram-negative types, as shown in Table I. The extracts showed no activity against *B. subtilis*, a Gram-positive spore-forming rod. The activity against *Pr. vulgaris*, *A. aerogenes*, and *Ps. aeruginosa*, three Gram-negative rods, remained fairly constant throughout the entire investigation as shown in Fig. 1. Figure 2 shows a comparison of the activity between *E. coli*, a Gram-negative rod,and *M. pyogenes* var. *aureus* and *M. pyogenes* var. *albus*, two Gram-positive cocci. The extracts showed favorable results against the four pathogenic organisms that were tested. Figure 3 gives a picture of this activity and shows the greatest inhibition recorded throughout the investigation. This was 3.2 cm. against *S. typhosa*, a short Gram-negative rod, during the month of September, 1952. A comparison between the activity of two Gram-negative pathogens, *Kl. pneumoniae* and *S. typhosa* and a Gram-negative potential, pathogen, *E. coli*, is shown in Fig. 4.

The activity of the extracts showed seasonal variation. The inhibition was greatest during the spring months and lowest during the winter season. The curves seem almost to level off during December and January.

The bacteriostatic and bactericidal effects of the extracts are shown in Table II. The extracts were

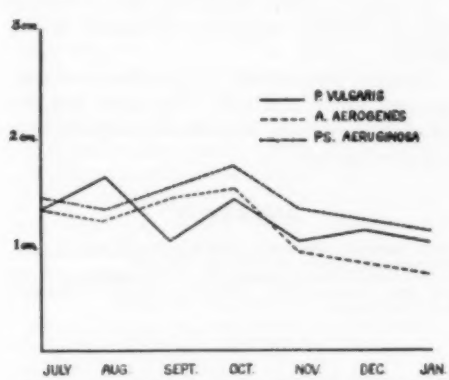

Fig. 1.—Inhibition curves for *Pr. vulgaris*, *A. aerogenes*, and *Ps. aeruginosa*. —*Pr. vulgaris*; ——*A. aerogenes*; -.-.-*Ps. aeruginosa*.

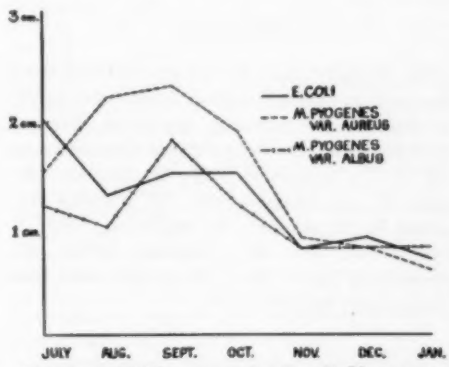

Fig. 2.—Inhibition curves for *E. coli*, *M. pyogenes* var. *aureus*, and *M. pyogenes* var *albus*. —*E. coli*; ——*M. pyogenes* var. *aureus*; -.-.-*M. pyogenes* var. *albus*.

TABLE II.—RESULTS OBTAINED FROM TESTING INHIBITON ZONES FOR BACTERICIDAL OR BACTERIOSTATIC ACTIVITY

Organisms Used	Growth in Nutrient Broth after 24-Hour Incubation at 37°C.						
	July	August	September	October	November	December	January
E. coli	+[a]	+	+	+	+	+	+
Ps. aeruginosa	+	−	+	+	+	+	+
M. pyogenes var. *albus*	−[b]	−	−	−	−	−	−
A. aerogenes	+	+	+	+	+	+	+
M. pyogenes var. *aureus*	+	+	+	+	+	+	+
Br. melitensis	−	−	−	−	−	−	−
S. typhosa	−	−	−	−	−	−	−
Kl. pneumoniae	−	−	−	−	−	−	−
Pr. vulgaris	−	−	−	−	−	−	−
S. pyogenes	+	+	+	+	+	+	+

[a] + indicates growth.
[b] − indicates no growth.

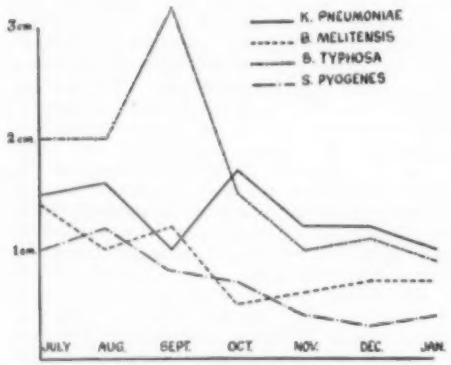

Fig. 3.—Inhibition curves for *Br. melitensis*, *Kl. pneumoniae*, *S. typhosa*, and *S. pyogenes*. —*Kl. pneumoniae*; ——*Br. melitensis*; -·-·-*S. typhosa*; ---*S. pyogenes*.

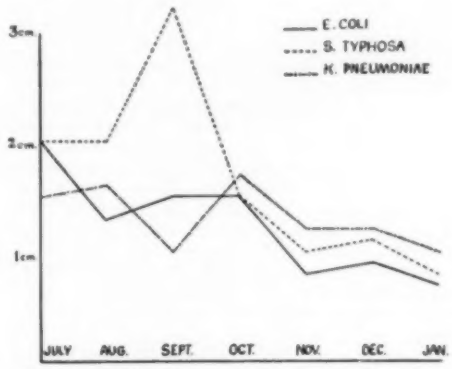

Fig. 4.—Inhibition curves for *E. coli*, *S. typhosa*, and *K. pneumoniae*. —*E. coli*; ——*S. typhosa*; -·-·-*Kl. pneumoniae*.

bactericidal against *M. pyogenes* var. *albus*, *Pr. vulgaris*, two nonpathogens, and *Br. melitensis*, *S. typhosa*, and *Kl. pneumoniae*, three pathogens. The activity was constant for the entire seven months with the exception of the extract from the August collection against *Ps. aeruginosa*.

SUMMARY

The monthly alcoholic extracts obtained from *Ascophyllum nodosum* showed antibacterial activity against the following organisms obtained from the American Type Culture Committee: *E. coli* 26, *Ps. aeruginosa* 9027, *M. pyogenes* var. *albus* 151, *A. aerogenes* 8308, *M. pyogenes* var. *aureus* F. D. A. 209, *Br. melitensis* 8360, *S. typhosa* F. D. A. Hopkins 6539, *Kl. pneumoniae* Type A 8045, *Pr. vulgaris* 8427, and *Streptococcus pyogenes* 4543.

The extracts showed no inhibition against *B. subtilis* a Gram-postive spore-forming rod. The activity was bactericidal against *M. pyogenes* var. *albus*, *Pr. vulgaris*, *Br. melitensis*, *S. typhosa*, and *Kl. pneumoniae*.

The results showed statistically that a marked seasonal variation existed in the activity of the extracts.

Chemical tests showed the presence of sodium, nitrogen, sulfur, and iron. Potassium and the halogens were absent, and there was a 39 per cent loss upon ignition.

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Studies on the Effects of Surface-Active Agents on the Extraction of Crude Drugs*

By EINAR BROCHMANN-HANSSEN†

Experiments were made to evaluate the fundamental aspects of various types of surface-active agents with a variety of structural differences, selected from anionic, cationic, and nonionic groups, on the extraction of crude drugs. It was found that the addition of a synthetic surface-active agent to an aqueous menstruum will tend to increase the rate of extraction of an alkaloidal crude drug. Anionic surface-active agents will precipitate alkaloids and are not suitable for extraction of alkaloidal drugs. Cationic surface-active agents in the form of salts with mineral acids show the best results.

THE EFFECTIVENESS of any extraction procedure for crude drugs is largely controlled by the permeability of the cell walls and the solubility of the desired constituents in the menstruum.

There is a considerable difference in the ease with which different drugs can be extracted. Büchi (1) has shown that among the commonly used alkaloidal drugs, cinchona and ipecac are the most difficult to extract. Nux vomica and hydrastis take an intermediate position, whereas the solanaceous drugs are rather easily extracted.

This difference in extractability is due in part to the different morphological condition of the cellular tissue, and in part to the difference in the solubility of the extractives.

Surface-active agents have properties which would indicate that they might be of value in the extraction of crude drugs. Wetting usually goes parallel with low surface tension and particularly with low interfacial tension. The characteristic surface tension curves for dilute solutions of surface-active agents, whether anionic, cationic, or nonionic, exhibit a steep decline. A tenth of one per cent is usually sufficient to lower the surface tension of water from 72 dynes/cm. to between 30 and 40 dynes/cm. Further increase in the concentration does not materially change the surface tension. Interfacial tension sometimes goes down to values of one dyne/cm. or less.

At or near the concentrations producing the minimum surface tension effects, many surface-active agents aggregate in the bulk of the solution to form colloidal micelles. The micelles have the property of causing a large increase in the solubility of many normally insoluble substances. This is presumed to be brought about by adsorption on or incorporation into the colloidal micelle. Solubilization, which this phenomenon is called, is already prominent at very low concentrations of the surface-active agent. Therefore, dilute solutions of surface-active agents can often take the place of a much more concentrated solution of an organic solvent.

It has also been shown that surface-active agents may carry solubilized materials through the finest of membranes, either to another surfactant or to or from a liquid in which the material is truly soluble (2).

EXPERIMENTAL

For the work reported here surface-active agents with a variety of structural differences were selected from each of the three main groups: anionic, cationic, and nonionic. Since these experiments were designed to evaluate fundamental aspects of various types of surface-active agents on the extraction, the compounds were chosen without any consideration for the toxicity or other undesirable properties. The products used are listed in Table I.

The crude drugs selected for this study were cinchona and ipecac. They were of uniform particle size, 22–30 mesh. In the case of ipecac, additional experiments were performed with a 30–40 mesh powder. The extractions were not carried out according to any official or conventional type of extraction procedure. In the hope of making the results more meaningful and easier to interpret the following simplified method was used: Ten grams of crude drug were placed in a 250-cc. prescription bottle and mixed with 100 cc. of the menstruum. The bottle was stoppered immediately and shaken in a mechanical shaker for a definite length of time, after which the content was rapidly filtered through a loose cotton plug into a dry flask. The filtrate was used for determination of surface tension, *p*H, and alkaloidal content. The following variables were controlled and their effects studied: (*a*) time of extraction, (*b*) type of surface-active agent, and (*c*) concentration of surface-active agent.

The temperature was maintained at 25±1° during the extraction.

Surface tension measurements were carried out by means of a Cenco-DuNoüy tensiometer, Model No.

* Received August 21, 1953, from the University of California College of Pharmacy, San Francisco, Calif.
Presented to the Scientific Section, A. PH. A., Salt Lake City Meeting, August, 1953.

† After the experimental work reported in this paper had been completed, W. J. Butler and G. A. Wiese described the effect of certain nonionic wetting agents on the preparation of fluidextracts of alkaloidal crude drugs [THIS JOURNAL, 42, 382(1953)].

TABLE I.—SURFACE-ACTIVE AGENTS USED FOR EXTRACTION[a]

Trade Name	Ionic Activity	Structural Classification	Manufacturer
Tween 20	nonionic	polyoxyethylene sorbitan monolaurate	Atlas Powder Co.
Tween 80	nonionic	polyoxyethylene sorbitan mono-oleate	Atlas Powder Co.
Antarox A-200	nonionic	alkaryl polyethylene glycol	Antara Products
Ethomid C/20	nonionic	polyoxyethylene substituted fatty acid amide	Armour and Co.
Duponol C	anionic	sodium alkyl sulfate	E. I. du Pont de Nemours & Co.
Ultrawet K	anionic	alkaryl sodium sulfonate	Atlantic Petroleum Chemicals
Cetyltrimethyl ammonium bromide	cationic	quaternary ammonium salt	Eastman Kodak
Hyamine 1622	cationic	quaternary ammonium salt	Rohm and Haas Co.
Ethomeen C/15	cationic	alkyl dipolyoxyethylene amine	Armour and Co.
Ethomeen C/25	cationic	alkyl dipolyoxyethylene amine	Armour and Co.

[a] The author is indebted to the manufacturers for supplying samples of the surface-active agents.

70530, and a Beckman model G *p*H meter was used for determination of *p*H.

The official assay procedures of the U. S. P. XIV and N. F. IX were not used in these experiments because they are too time consuming where a large number of determinations are required. Furthermore, a gravimetric method, such as the official assay method for cinchona, is not suitable in the presence of surface-active agents which have an appreciable solubility in organic solvents. The procedures used were, however, found to be of at least equal accuracy and permitted a considerable saving of time. Cinchona was assayed for total alkaloids according to the Norwegian Pharmacopeia (1939), whereas the procedure of the Danish Pharmacopeia (1948) was used for determination of the ether-soluble alkaloids of ipecac. Adaptations of these methods were applied to the extracts of the two drugs. Experiments were carried out with known amounts of pure alkaloids in order to find out if the assay procedures could be used in the presence of the surface-active agents. The extraction of the alkaloid bases with the organic solvent or solvent mixture was found to be complete in every case. A few of the surfactants gave small, but still significant blank values which were considered in the calculation of the results. Only in the case of the Ethomeens were the blank values so large as to make a volumetric method impractical. A spectrophotometric procedure was, therefore, used for extracts prepared with these surface-active agents. The total alkaloid from cinchona, when dissolved in ethanol, gave absorption maxima at 332 mμ, 315 mμ, 302 mμ, and 285 mμ. The first maximum is entirely caused by the quinine-quinidine fraction, the next two are mainly due to the cinchonine-cinchonidine fraction, whereas the last one (285 mμ) is caused jointly by both fractions. The presence of small amounts of the Ethomeens did not have any effect on the absorption curves, which were obtained by means of a Cary Recording Spectrophotometer, Model 11 PMS. Extracts of cinchona prepared with various concentrations of the Ethomeens gave absorption curves in which the ratio between the optical densities at 332 mμ and at 285 mμ remained fairly constant. This indicated that both alkaloid fractions were extracted equally well, making it possible to use the optical density at 285 mμ for calculation of total cinchona alkaloids. From a known amount of total alkaloid extracted from the same sample of crude drug, the extinction coefficient at this wave length was found to be 4,684, when an average value of 309 was used as the molecular weight.

RESULTS AND DISCUSSION

Nonionic Surface-Active Agents.—The results of the extraction of cinchona with various concentrations of Antarox A-200 are given in Table II. Similar results were obtained with the other nonionic surface-active agents used. The effect of Tween 80 on the extraction of cinchona is illustrated in Fig. 1.

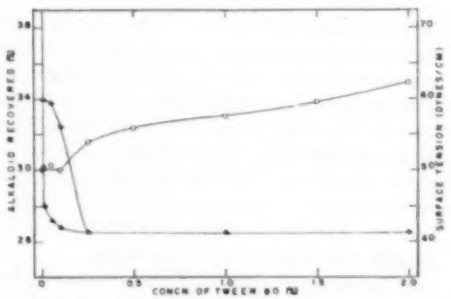

Fig. 1.—Extraction of cinchona with aqueous solutions of Tween 80 (-O-O-). Surface tension of extracts (-Δ-Δ-) and menstrua (-▲-▲-). Extraction time 30 min.

A relatively high concentration of nonionic surface-active agents is necessary in order to produce a significant increase in extraction efficiency. This is due, at least in part, to the fact that the surfactants are in some way removed from solution, probably by adsorption on the surface of the crude drug, as indicated by the slow decline in the surface tension curve for the extracts (Fig. 1). From Table II it is

TABLE II.—EXTRACTION OF CINCHONA WITH AQUEOUS SOLUTIONS OF ANTAROX A-200 (RESULTS ARE EXPRESSED AS PER CENT OF TOTAL ALKALOID)

Time of Extraction	Concentration of Antarox A-200 in Menstruum, %							
	0.00	0.01	0.05	0.10	0.25	0.50	1.00	2.00
15 min.	27.5	...	...	26.8	...	29.4	...	...
30 min.	29.9	29.7	30.1	30.0	31.2	33.2	34.7	36.3
1 hr.	31.9	...	...	31.9	...	35.0	...	...
2 hr.	33.4	...	...	32.2	...	36.3	...	...
4 hr.	32.9	...	...	33.8	...	36.5	...	...
8 hr.	32.7	...	...	33.4	...	36.0	...	...
24 hr.	33.2	...	...	33.7	...	36.7	...	...
48 hr.	33.5	...	...	33.8	...	37.0	...	...

seen that an equilibrium is reached in the same short time (about two hours) with 0.5% of Antarox A-200 as with water alone. This may lead one to the conclusion that the increased rate of extraction observed may not be due to an increase in the penetration of the drug particles, but may rather be caused by solubilization of alkaloid from easily accessible surface cells.

The extraction of ipecac with plain water gave a surprisingly high recovery of alkaloids which was not significantly increased in the presence of a nonionic surface-active agent (Table III). Such an aqueous extract was found to have a very low surface tension, about 40 dynes/cm. as compared to about 60 dynes/cm. for the corresponding cinchona extract. This low surface tension is probably due to the presence of ipecacuanhin, a glycoside with saponin character, which has been found to exist in ipecac (3). It seems reasonable to believe that this glycoside is at least to some extent responsible for the high yield obtained with water.

The nonionic surface-active agents seemed to have a less significant effect on the extraction of cinchona when dissolved in alcohol than when dissolved in water (Table IV). Alcohol is a better solvent for the alkaloid-tannin complexes than water, and addition of a surfactant apparently does not appreciably increase the effectiveness of alcohol as an extraction medium under the conditions of the experiments described in this paper.

Anionic Surface-Active Agents.—Surface-active agents of the anionic type form rather insoluble precipitates with high molecular weight cations, like the alkaloids, resulting first in a decrease in the yield of extraction. As the concentration of surface-active agent is increased, a point is reached where the precipitate begins to go into solution by solubilization. At a concentration of 3% of sodium lauryl sulfate the recovery of cinchona alkaloids was almost 40% in excess of that obtained with water alone.

Sodium lauryl sulfate gave a somewhat different curve with ipecac (*cf.* Table III) than with cinchona. Although a precipitate was formed with the alkaloids of ipecac and sodium lauryl sulfate, this precipitate was so finely divided that it passed through the cotton filter and was, therefore, included in the assay. When the concentration of sodium lauryl sulfate reached 0.5%, the precipitate apparently flocculated and was removed during the filtration. At higher concentrations, on the other hand, the precipitate was solubilized. A low recovery of ipecac alkaloid was, therefore, only observed at concentrations of sodium lauryl sulfate in the neighborhood of 0.5%.

Addition of sodium lauryl sulfate to pure solutions of quinine hydrochloride, as well as to aqueous extracts of cinchona, resulted in surface tension curves which indicated that the quinine lauryl sulfate com-

TABLE III.—EXTRACTION OF IPECAC WITH AQUEOUS SOLUTIONS OF SURFACE-ACTIVE AGENTS (RESULTS ARE EXPRESSED AS PER CENT OF TOTAL ALKALOID; EXTRACTION TIME 30 MIN.)

Concentration of Surface-Active Agent, %	Type of Surface-Active Agent					
	Antarox A-200		Tween 80	Duponol C		C. T. A. B.
	22–30[a]	30–40[a]	22–30[a]	22–30[a]	30–40[a]	30–40[a]
0.00	55.3	76.5	55.9	55.1	75.4	75.4
0.01	54.7	76.5	56.1	55.0	75.4	75.4
0.05	55.9	77.1	56.5	55.6	76.5	75.9
0.10	55.3	75.4	54.7	56.2	75.1	76.5
0.25	55.9	76.7	56.1	55.1	77.0	80.1
0.50	55.9	76.5	55.8	16.9	31.3	82.7
1.00	56.3	76.0	55.5	61.1	78.6	84.6
2.00	56.9	77.5	56.8	64.0	90.5	88.5

[a] Range of sieve sizes indicating the particle size of ipecac.

TABLE IV.—EXTRACTION OF CINCHONA WITH ALCOHOLIC SOLUTIONS OF ANTAROX A-200 (RESULTS ARE EXPRESSED AS PER CENT OF TOTAL ALKALOID; EXTRACTION TIME 30 MIN.)

Concentration of Antarox A-200	Concentration of Alcohol in Solvent	
	70% V/V	10% V/V
0.00	58.5	30.6
0.01	58.7	30.4
0.05	59.0	30.6
0.10	59.2	30.6
0.25	58.5	30.2
0.50	59.7	30.9
1.00	59.2	32.2
2.00	60.0	33.9

TABLE V.—EXTRACTION OF CINCHONA WITH AQUEOUS SOLUTIONS OF CETYLTRIMETHYLAMMONIUM BROMIDE (RESULTS ARE EXPRESSED AS PER CENT OF TOTAL ALKALOID)

Time of Extraction	Concentration of C. T. A. B. in Menstruum, %							
	0.00	0.01	0.05	0.10	0.25	0.50	1.00	2.00
15 min.	27.2	...	...	31.7	...	36.5	...	...
30 min.	30.1	30.9	31.7	34.2	37.1	41.6	43.9	47.8
1 hr.	31.6	...	...	36.5	...	43.9	...	...
2 hr.	32.7	...	...	37.0	...	46.7	...	...
4 hr.	32.9	...	...	37.5	...	46.7	...	...
8 hr.	32.4	...	...	37.0	...	48.5	...	...
24 hr.	32.2	...	...	36.9	...	49.0	...	...
48 hr.	32.9	...	...	37.6	...	48.5	...	...

plex probably has a greater surface activity in low concentrations than the sodium salt. However, the solubility of this complex is low. The surface tension will, therefore, remain constant as long as there is an excess of quinine in solution (Fig. 2). The difference between the surface tension curve representing extracts prepared with sodium lauryl sulfate solutions and that representing aqueous cinchona extracts to which sodium lauryl sulfate had been added may be explained as being due to adsorption.

Sodium lauryl sulfate does not cause any appreciable change in the *p*H of the cinchona extracts except in high concentrations (> 2%). This shows that the crude drug with its content of organic acids has a considerable buffer capacity.

Cationic Surface-Active Agents.—The surface-active agents of the quaternary ammonium type increase the yield of cinchona alkaloids even in very low concentrations. The relative slowness with which equilibrium is attained further indicates that the permeability of the cells may have been affected. The results of the extraction of cinchona with cetyltrimethylammonium bromide are found in Table V. Similar results were given by Hyamine 1622.

The effect on the extraction of ipecac is less pronounced, but still significant (*cf.* Table III).

As may be expected, no precipitate is formed between quinine and a quaternary surface-active agent. However, if this type of surfactant is added to an aqueous extract of cinchona, precipitation takes place. This indicates that an ion exchange reaction occurs, forming rather insoluble complexes between the quaternary cation and the organic acids from the crude drug. This ion exchange mechanism may explain why the quaternary surface-active agents are much more effective in increasing the extraction yield than are those of the nonionic type, as illustrated in the following reaction:

$$R_4N^+X^- + \text{Alkaloid-tannin complex} \rightarrow R_4N\text{-tannin complex} + \text{Alkaloid}^+ X^-$$

By the ion exchange postulate, it is assumed that the alkaloids are converted from rather insoluble complexes with organic acids to very soluble salts of inorganic acids.

The tertiary amine type of surface-active agents, when used in the form of the free bases, had a much less pronounced effect on the extraction than did the quaternaries (Table VI). They resembled more the nonionic type in their effect. The Ethomeens did not change the *p*H of the cinchona extracts appreciably unless used in rather high concentrations (Table VII). This shows that the surface-active agents are being neutralized by the organic acids in the crude drug.

If the Ethomeens are used in the form of hydrochlorides by partial or complete neutralization with hydrochloric acid, their effect on the extraction of cinchona is greatly enhanced (Fig. 3). This is not due to the presence of chloride ions or to a lowering of *p*H, but rather to an ion exchange reaction as postulated for the quaternary ammonium salts. This conclusion is based on the observation that mixtures of ammonium chloride and ammonium hydroxide, containing the same chloride ion concentra-

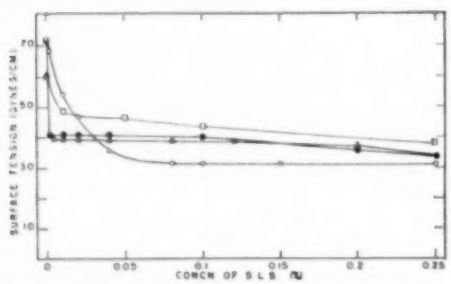

Fig. 2.—A comparison between surface tension curves obtained with S. L. S. solutions (-O-O-), cinchona extracts prepared with S. L. S. solutions (-□-□-), aqueous cinchona extracts with S. L. S. added (-Δ-Δ-), and quinine hydrochloride solutions with S. L. S. added (-●-●-).

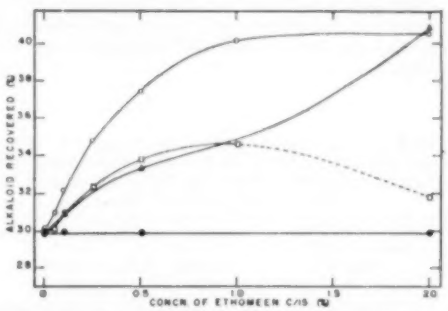

Fig. 3.—Extraction of cinchona with aqueous solutions of (*a*) Ethomeen C/15 (-□-□-), (*b*) Ethomeen C/15 half neutralized with HCl (-O-O-), (*c*) $NH_4Cl + NH_3$ mixtures (-●-●-), (*d*) hydrochloric acid (-Δ-Δ-); (*c*) and (*d*) contain concentrations of Cl^- corresponding to those found in (*b*).

TABLE VI.—EXTRACTION OF CINCHONA WITH AQUEOUS SOLUTIONS OF ETHOMEEN C/25 (RESULTS ARE EXPRESSED AS PER CENT OF TOTAL ALKALOID)

Time of Extraction	Concentration of Ethomeen C/25 in Menstruum, %							
	0.00	0.01	0.05	0.10	0.25	0.50	1.00	2.00
15 min.	27.2	...	...	27.9	...	30.3	...	...
30 min.	29.9	29.9	30.5	31.5	32.6	34.8	35.6	36.0
1 hr.	31.2	...	...	32.0	...	36.6	...	...
2 hr.	31.6	...	...	33.4	...	38.2	...	...
4 hr.	32.3	...	...	33.9	...	38.0	...	...
8 hr.	31.6	...	...	33.5	...	39.0	...	...
24 hr.	31.8	...	...	33.9	..	38.8	...	...
48 hr.	32.6	...	...	34.1	...	39.5	...	...

TABLE VII.—*p*H OF AQUEOUS SOLUTIONS OF ETHOMEEN C/15 AND ETHOMEEN C/25 AS WELL AS OF AQUEOUS EXTRACTS OF CINCHONA PREPARED WITH THESE SURFACE-ACTIVE AGENTS

Concentration of Surfactant	Ethomeen C/15		Ethomeen C/25		Ethomeen C/15—Half Neutralized with HCl	
	Menstruum	Extract	Menstruum	Extract	Menstruum	Extract
0.0	6.2	4.0	6.2	4.0	6.2	4.0
0.01	8.1	4.0	7.7	4.0	6.5	4.0
0.05	8.7	4.0	8.6	4.0	6.8	4.0
0.10	8.9	4.1	8.9	4.0	6.7	4.0
0.25	9.2	4.2	9.0	4.1	...	4.0
0.50	9.4	4.3	9.1	4.2	6.5	4.1
1.00	9.6	4.8	9.3	4.4	...	4.2
2.00	9.8	6.1	9.4	4.9	6.4	4.7

tion and having approximately the same *p*H as the corresponding concentrations of half neutralized Ethomeen C/15, had no effect on the extraction of cinchona. The corresponding concentrations of hydrochloric acid, however, gave a considerable increase in the extraction yield (Fig. 3).

The decrease in recovery of alkaloid observed with 2% solution of Ethomeen C/15 may have some connection with the marked change in the *p*H of the extract at this point (*cf.* Table VII), possibly resulting in precipitation of some alkaloid in the form of the base.

Adsorption of the Ethomeens on the surface of the crude drugs is appreciable and is apparently not affected by neutralization.

CONCLUSIONS

Although the experimental work reported in this paper is not sufficient to allow definite conclusions to be drawn, there is evidence that addition of a synthetic surface-active agent to an aqueous menstruum will tend to increase the rate of extraction of an alkaloidal crude drug. The effect appears to be due mainly to solubilization and, in the case of cationic surface-active agents, to ion exchange. The surfactants are very readily adsorbed on the surface of the crude drugs. Their effectiveness as solubilizing agents is thereby greatly reduced, making it necessary to use rather high concentrations in order to obtain an appreciable effect. Alcoholic solutions of nonionic surface-active agents showed little or no increase in yield over that obtained with the solvent alone.

Anionic surface-active agents will precipitate alkaloids and are, therefore, not suitable for extraction of alkaloidal drugs, except possibly in high concentrations where solubilization of the precipitate takes place. Cationic surface-active agents in the form of salts with mineral acids show the best results. This is assumed to be due to ion exchange, resulting in the formation of highly soluble alkaloidal salts.

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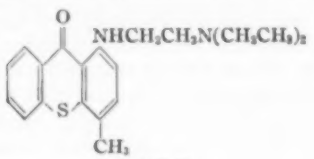
Studies on the Chemotherapy of Experimental Schistosomiasis. II. N-(9-Xanthenyl)amides*

By HOWARD W. BOND and GEORGE W. LUTTERMOSER†

Thirty-five N-(9-xanthenyl)amides have been prepared and tested for their activity against experimentally induced *Schistosoma mansoni* in Swiss albino mice. Miracil D has been used as the standard of reference. Ten of the compounds show some activity. None, however, appears to be as active in mice as Miracil D.

SINCE THE DISCOVERY by Kikuth and coworkers (1, 2) of the activity of certain dialkylaminoalkylamino derivatives of xanthone and of thiaxanthone against *Schistosoma mansoni*, many closely related compounds have been prepared and tested for their activity. Mauss (3) prepared and Kikuth tested 45 derivatives of xanthone and 28 derivatives of thiaxanthone against *S. mansoni* in mice and Rhesus monkeys, finding the most active compound to be 1-(diethylaminoethylamino)-4-methylthiaxanthone (Miracil D).



Miracil D

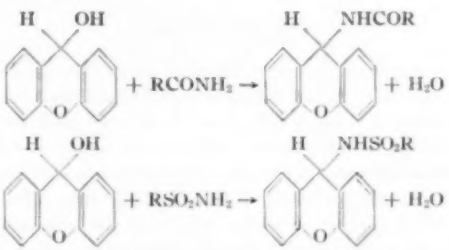
Miracil D has since been subjected to extensive clinical testing in humans.

Nearly all of the derivatives prepared by Mauss contained the side chain in the 1-position. Mann and Turnbull (4) prepared five derivatives of xanthone and seven of thiaxanthone having the dialkylaminoalkylamino group in the 2- and 3-positions. All were found by Standen (see (5) for the technique employed) to be ineffective against *S. mansoni* in white mice. Sharp (6) prepared four derivatives of thiaxanthone similar to the Miracils prepared by Mauss, i. e., having the side chain in the 1-position. All were found by Standen to be less active than Miracil D against *S. mansoni* in white mice. Archer and Suter (7) have recently reported on 76 compounds in the 1-(alkylaminoalkylamino) thiaxanthone series, 68 of them being compounds whose activities had not previously been described. A number of these derivatives, particularly those containing a hydroxyl group in the terminal alkyl group(s), are indicated by Berberian and co-workers as having high therapeutic indices against *S. mansoni* in white mice and therefore as being of considerable promise as schistosomicidal agents.

Mann and Reid (8) have prepared 4-azaxanthone and 4-azathiaxanthone. Standen has found, as would be predicted, that these compounds, lacking as they do a side chain, have no significant effect on *S. mansoni* in mice.

Although numerous preparations of xanthene derivatives having substituents in the 9-position are recorded in the literature, seemingly no such compounds have been tested for their schistosomicidal activity. Mann and Turnbull (9) prepared 9-xanthenylamine and 9-thiaxanthenylamine with the intent of using them as starting materials for the synthesis of 9-dialkylaminoalkylamino derivatives. They discontinued work along these lines when they found these two amines to be unstable, even at room temperature.

The ease of preparation of N-(9-xanthenyl)-amides from xanthydrol and amides in acetic acid solution at room temperature suggested that a number of these be prepared and tested. The reaction works equally well for carboxamides and for sulfonamides.



A number of such derivatives have been described in the literature, but to date no pharmacological data appear to be recorded for any of them.[1] In the present study, 26 carboxamide derivatives and nine sulfonamide derivatives of xanthydrol have been prepared and tested. Five of the former and four of the latter are new

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† The authors wish to thank the Microanalytical Laboratory of the National Institutes of Health for the chemical analyses included herein and to acknowledge the assistance of Otis L. Kline and Wesley G. Blodgett in the biological portion of this study.

[1] Schubert (10) found xanthydrol to be ineffective against *S. mansoni* in white mice.

compounds. A number of the derivatives show marked activity against *S. mansoni* in Swiss albino mice.

EXPERIMENTAL

Preparation of Compounds.—The general procedures of Phillips and Pitt (11) for the preparation of N-(9-xanthenyl)carboxamides and of Phillips and Frank (12) for the preparation of N-(9-xanthenyl)sulfonamides were followed. All recrystallizations were from 65% aqueous dioxan. Satisfactory analytical values were obtained in all cases, with the exception of the cyanoacetamide and the methyl urea derivatives. Only the preparation of 9-(di-*n*-propylaminoacetamido)xanthene need be given in detail here.

9-(Di-*n*-propylaminoacetamido)xanthene.—A mixture of 2.74 Gm. (0.01 mole) of 9-(chloroacetamido)xanthene and 3.04 Gm. (0.03 mole) of di-*n*-propylamine in 50 cc. of toluene (dried over sodium) was refluxed for twenty-four hours. A small amount of solid formed when the mixture was cooled to room temperature. Without being filtered, the mixture was transferred to a separatory funnel by means of 50 cc. of 1 *N* hydrochloric acid. Three layers formed in the funnel. The bottom oily layer and the intermediate water layer were removed, and the mixture of the two was made basic with 5% aqueous sodium hydroxide. An oil separated which soon solidified. This solid was filtered off, washed with water, and dried in the air, yielding 3.30 Gm. (98%) of yellowish-brown, granular product, m. p. 55.5–57.0°. One recrystallization from 20 ml. of 50% aqueous ethanol gave 2.93 Gm. of tan product, m. p. 55.5–57.0°. A second recrystallization from 20 ml. of 95% ethanol gave 2.77 Gm. of white crystalline material, m. p. 81.5–82.5°. When the melting-point sample was allowed to cool to room temperature and was then reheated, it melted at 55°, resolidified on further heating, and melted again at 81.5–82.5°. This would indicate that the product occurs in two crystalline modifications.

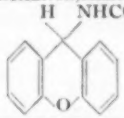
Anal.—Calcd. for $C_{21}H_{26}N_2O_2$: C, 74.53; H, 7.74. Found: C, 74.26; H, 7.69.

Biological Results.—The maximum tolerated dose of the compounds in infected Swiss albino mice was found to range from 25 to 200 mg./Kg. of body weight when administered intraperitoneally twice daily for five consecutive days. However, since many of the compounds remained unabsorbed in the body cavity for more than a week, their toxicities could not be accurately determined. The schistosomicidal activity of the compounds given intraperitoneally twice daily for five days at the dosage level indicated in the tables was determined by the method described by Luttermoser (13, 14). The main criteria for activity were the presence of dead or degenerate schistosomes in the livers of treated mice and the increase of survival time of animals given a lethal infection of *S. mansoni*. The following arbitrary scale of activity was adopted: −, inactive at the level tested; ±, questionable activity; +, very slight activity; ++, slight activity; +++, activity approximating that of Miracil D in mice; ++++, activity equal to that of Miracil D in mice.

RESULTS

The results are listed in Tables I, II, and III. Four of the alkylcarboxamide derivatives had activities comparable with that of Miracil D. These were the stearamide, the chloroacetamide, the *n*-propyl carbamate, and the *n*-butyl carbamate derivatives. None of the arylcarboxamide derivatives showed any activity. None of the arylsulfonamide derivatives showed any activity, but the α-toluenesulfonamide derivative was as active as any of the

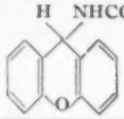
TABLE I.—N-(9-XANTHENYL)ALKYLCARBOXAMIDES



R	M.P., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Dose (Mg./Kg.)	Activity
CH_3	238.0–238.5	$C_{15}H_{13}NO_2$	75.28	75.44	5.48	5.75	100	−
C_2H_5	209.0–210.0	$C_{16}H_{15}NO_2$	75.88	75.90	5.97	5.97	100	−
n-C_3H_7	181.5–182.5	$C_{17}H_{17}NO_2$	76.38	76.52	6.41	6.37	200	±
n-C_4H_9	168.5–169.5	$C_{18}H_{19}NO_2$	76.84	76.92	6.81	6.97	200	−
iso C_4H_9	182.0–182.4	$C_{18}H_{19}NO_2$	76.84	76.59	6.81	7.04	100	−
n-$C_{17}H_{35}$	138.0–140.0	$C_{31}H_{49}NO_2$	80.30	80.29	9.78	9.87	50	+++
CH_2=CH	232.5–233.0	$C_{16}H_{13}NO_2$	76.48	76.57	5.21	5.42	50	+
C_2H_5CH=CH	214.5–215.0	$C_{19}H_{17}NO_2$	77.39	77.12	6.14	6.36	150	++
$ClCH_2$	205.0–206.0	$C_{16}H_{12}NO_2Cl$	65.81	65.93	4.42	4.68	100	+++
$CNCH_2$	221.5–222.5	$C_{16}H_{12}N_2O_2$	72.71	71.13	4.58	5.46	200	+
C_2H_5O	164.5–165.0	$C_{18}H_{19}NO_3$	71.37	71.41	5.62	5.59	150	++
n-C_3H_7O	141.5–142.0	$C_{17}H_{17}NO_3$	72.06	71.91	6.05	6.02	150	+++
n-C_4H_9O	139.5–140.0	$C_{18}H_{19}NO_3$	72.71	72.92	6.44	6.46	150	+++
$(n\text{-}C_3H_7)_2NCH_2$	81.5–82.5[a]	$C_{21}H_{26}N_2O_2$	74.53	74.26	7.74	7.66	50	−
$(CH_2)_4$[b]	258.0–259.5	$C_{32}H_{28}N_2O_4$	76.17	76.40	5.59	5.67	50	±
CH_3NH	227.0–230.5	$C_{15}H_{14}N_2O_2$	70.84	71.74	5.55	5.61	100	−
$C_6H_5CH_2$	192.5–193.5	$C_{21}H_{17}NO_2$	79.98	79.86	5.44	5.38	200	−
Miracil D	...	...	...	...	...	...	50	++++

[a] See experimental part.
[b] Bis(adipamide) derivative.

TABLE II.—N-(9-XANTHENYL)ARYLCARBOXAMIDES



Ar	M. P., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Dose (Mg./Kg.)	Activity
C_6H_5	220.5–221.0	$C_{20}H_{13}NO_2$	79.73	79.81	5.02	5.21	200	−
2-CH_3-C_6H_4	198.0–200.0	$C_{21}H_{17}NO_2$	79.98	79.79	5.44	5.64	200	−
4-CH_3-C_6H_4	223.0–224.0	$C_{21}H_{17}NO_2$	79.98	79.76	5.44	5.26	200	−
4-NO_2-C_6H_4	225.5–229.5	$C_{20}H_{14}N_2O_4$	69.36	69.26	4.07	3.98	100	−
C_4H_3O[a]	207.0–209.5	$C_{18}H_{13}NO_3$	74.22	74.27	4.50	4.65	200	±
Phthalimido	178.5–179.0	$C_{21}H_{12}NO_3$	77.06	77.23	4.00	4.05	50	−
4-NO_2-Phthalimido	185.0–186.5	$C_{21}H_{12}N_2O_5$	67.75	67.88	3.25	3.28	100	−
Succinimido	242.0–243.5	$C_{17}H_{13}NO_3$	73.11	73.23	4.69	4.95	200	−
o-Benzoic sulfimido	193.0–193.5	$C_{20}H_{13}NO_4S$	66.10	65.98	3.61	3.82	100	−
Miracil D	...	...	...	...	...	...	50	++++

[a] Furyl.

compounds tested. It will be noted that the name of this compound is misleading; the compound is actually an alkylsulfonamide. Slight activity was shown by the β-ethylacrylamide and the ethyl carbamate derivatives. Of the active compounds, only the chloroacetamide and the α-toluenesulfonamide derivatives prolonged the survival time of the mice.

DISCUSSION

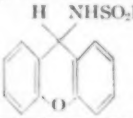
Previous investigators have shown that particular side chains in the 1- and 4-positions are necessary for activity in the xanthones and thiaxanthones. In the present study, the substituents were in the 9-position. Activity among these compounds was therefore dependent upon the groupings in this position. It is possible that their activity is due to their hydrolysis within the test animal to 9-xanthenylamine, which may well be the active compound. It is interesting to note that active compounds were found only among the alkylcarboxamide, alkylsulfonamide, and carbamate derivatives.

The encouraging results obtained with these compounds suggest the preparation of additional analogs including those bearing a methyl group in the 4-position of the xanthone ring, as in Miracil D.

SUMMARY

Seventeen N-(9-xanthenyl) alkylcarboxamides, nine N-(9-xanthenyl)arylcarboxamides, and nine N-(9-xanthenyl)sulfonamides have been prepared and tested for their activity against *Schistosoma mansoni* infections in Swiss albino mice. Four of the alkylcarboxamide derivatives, none of the arylcarboxamide, and one of the sulfonamide derivatives showed activity comparable to Miracil D. None, however, appeared to be as active as Miracil D. Two of the compounds the chloroacetamide and the α-toluenesulfonamide derivatives, prolonged the survival time of the mice.

TABLE III.—N-(9-XANTHENYL)SULFONAMIDES



R	M. P., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Dose (Mg./Kg.)	Activity
C_6H_5	196.0–197.0	$C_{19}H_{15}NO_3S$	67.64	67.86	4.48	4.37	50	−
2-CH_3-C_6H_4	185.5–186.5	$C_{20}H_{17}NO_3S$	68.36	68.43	4.88	5.06	25	−
4-CH_3-C_6H_4	195.5–196.0	$C_{20}H_{17}NO_3S$	68.36	68.62	4.88	5.06	25	±
2,5-$(CH_3)_2$-C_6H_3	178.0–181.0	$C_{21}H_{19}NO_3S$	69.02	69.21	5.24	5.34	100	±
4-NO_2-C_6H_4	215.5–216.0	$C_{19}H_{14}N_2O_5S$	59.68	59.68	3.69	3.79	50	−
4-Br-C_6H_4	205.5–207.0	$C_{19}H_{14}NO_3BrS$	54.82	54.76	3.39	3.44	100	−
4-Cl-C_6H_4	204.5–205.5	$C_{19}H_{14}NO_3ClS$	61.38	61.54	3.80	3.81	100	−
$C_6H_5CH_2$	178.0–179.0	$C_{20}H_{17}NO_3S$	See footnote [a] below				25	+++
2-$C_{10}H_7$[b]	209.0–209.5	$C_{23}H_{17}NO_3S$	71.30	71.32	4.42	4.63	100	+
Miracil D	...	...	...	...	...	...	50	++++

[a] This compound crystallized from aqueous dioxan as $C_{20}H_{17}NO_3S$·½$C_4H_8O_2$. Calcd.: C, 66.82; H, 5.35; N, 3.54. Found: C, 66.78; H, 5.41; N, 3.71.
[b] 2-Naphthyl.

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The Newer Anticholinergic Agents. I. Effectiveness as Anhydrotics*

By ARTHUR G. ZUPKO and LEON D. PROKOP†

Pro-Banthine, Antrenyl, Pamine, and Darstine are compared with Banthine and Prantal as to their effectiveness in reducing sweat in hidrose subjects and in normal subjects in whom hyperhidrosis was induced. Results of quantitative determinations on various parts of the body before and after drug or placebo administration are discussed. The effects of prolonged administration and side effects of the drugs are reported.

INCREASED INTEREST in the anticholinergic drugs has been manifested since the introduction of Banthine in May, 1950. The desire for greater efficiency of action with minimal or no side effects has resulted in the synthesis of many promising compounds, among which may be listed Prantal, Antrenyl, Pro-Banthine, Pamine, and Darstine. Figure 1 shows the chemical configurations of these compounds. The anticholinergic drugs are particularly efficacious for the symptomatic relief of conditions which may be benefited by interruption of cholinergic transmission, especially peptic ulcers. Previous studies on Banthine and Prantal (1–4) have indicated that these agents are also effective in hyperhidrosis.

Hyperhidrosis occurs with far greater frequency than is generally believed. Inquiry among many dermatologists and general practitioners reveals that an estimated one to one and one-half million people in the United States may be classified as hidrose individuals (5). Though neither serious nor debilitating, hyperhidrosis nevertheless is an extremely annoying condition and upon occasion may cause much social embarrassment. Uncontrolled hyperhidrosis may also aggravate certain dermatoses. In an effort to ascertain the effectiveness of these newer anticholinergic agents in hyperhidrosis, both qualitative and quantitative studies of their anhydrotic effects were undertaken. Note was made also of side effects resulting from daily administration of the drugs to large groups of hidrose and normal subjects over a prolonged period.

EXPERIMENTAL

Methods.—Pro-Banthine, Antrenyl, Pamine, and Darstine were administered in varying dosages to test groups of hidrose and normal subjects over periods ranging from six weeks to eight months. In general, prior to administration of each drug, a medical history was obtained and the subject given an information sheet with ample directions regarding administration and admonition of side effects. The subject reported each week for an additional supply of the drug and related his experience with the medication for the previous week. If the medication proved to be ineffective or resulted in a pronounced side effect, another anticholinergic drug was substituted. In each case an attempt was made to titrate the subject for effective dosage to control the hyperhidrosis. Pertinent data on the test subjects including the range of dosages utilized for hidrose and normal subjects as well as the duration of the test period for each of the drugs are shown in Table I. Only those subjects who participated in the experiment for a minimum of six weeks are included.

Similar test groups were used for quantitative determinations of sweat on various parts of the body

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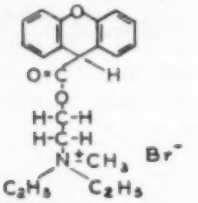
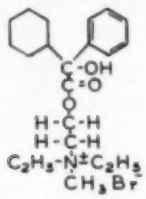
BANTHINE (Searle)



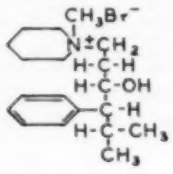
Beta-diethylaminoethyl xanthene 9-carboxylate methobromide

ANTRENYL (Ciba)



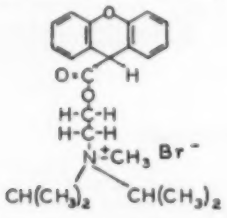
Diethyl (2-hydroxyethyl) methyl ammonium bromine-phenyl-cyclohexaneglycolate

DARSTINE (Sharp & Dohme)



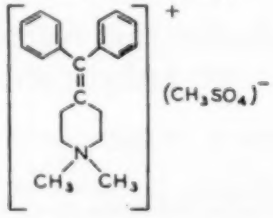
5 - methyl - 4 - phenyl - 1-(1 - piperidyl) - 3 - hexanol methobromide

PRO-BANTHINE (Searle)



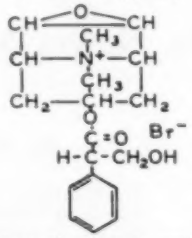
Beta - diisopropylaminoethyl-xanthene 9 - carboxylate methobromide

PRANTAL (Schering)



N,N - dimethyl - 4 -piperidyl-idene - 1,1 - diphenylmeth-anil methyl sulfate

PAMINE (Upjohn)



Epoxytropine tropate methyl bromide

Fig. 1.—Chemical Configurations of the Anticholinergic Drugs.

prior to and after drug or placebo administration. Both normal and hidrose subjects were exposed to thermostatically controlled infrared heating panels mounted in a constant temperature room. Experiments were conducted at consistently high dry bulb temperatures of 91–97° F. with relative humidity variations of 43 to 81%. For the collection of sweat a method previously described was utilized (4). This involved the use of a tared unit consisting of activated alumina and absorbent blotting paper within an aluminum cup or 1/3 oz. ointment tin. The change in weight of the prepared unit after removal from a body area represented the amount of sweat secreted during the test period. A majority of the subjects received a dosage equivalent to twice that of the recommended single oral dose of each drug; others were administered placebo tablets. In some instances, in order to evaluate further the comparative efficiency of sweat inhibition for each drug, quantitative determinations were performed on four separate days on the same individual using a different anticholinergic drug on each occasion.

Blind studies, in which neither operator nor subject knew the identity of the drug used, were conducted throughout the course of these experiments.

RESULTS

Effect on Hyperhidrosis.—One hundred and sixteen hidrose subjects were arbitrarily classified as individuals with mild, pronounced, or severe hyperhidrosis, depending upon the severity of the symptoms. Table II shows the effectiveness of the drugs under study in controlling hyperhidrosis. A comparison is also made of results reported in previous studies with Banthine and Prantal (3, 4).

TABLE I.—PERTINENT DATA ON TEST SUBJECTS

	Pro-Banthine	Antrenyl	Pamine	Darstine
Hidrose subjects	39	37	22	18
Normal subjects	8	5	4	4
Average age (years)	24.7	25.1	23.7	26.3
Sex	38 M, 9 F	36 M, 6 F	22 M, 4 F	17 M, 5 F
Hidose dosage mild	15 mg. t. i. d. or 30 mg. b. i. d.	5 mg. t. i. d. or 10 mg. b. i. d.	2.5 mg. t. i. d. or 5 mg. b. i. d	50 mg. t. i. d. or 100 mg. b. i. d.
Pronounced	30 mg. t. i. d.	10 mg. t. i. d.	5 mg. t. i. d.	100 mg. t. i. d.
Severe	30 mg. t. i. d.–q. i. d.	10–15 mg. t. i. d.–q. i. d.	5–7.5 mg. t. i. d.–q. i. d.	100–150 mg. t. i. d.–q. i. d.
Normal or control dosage	15 mg. t. i. d.	5 mg. t. i. d.	2.5 mg. t. i. d.	50 mg. t. i. d.
Minimum duration of test period	6 weeks	6 weeks	6 weeks	6 weeks
Maximum duration of test period	8 months	5 months	3 months	9 weeks

TABLE II.—EFFECTIVENESS OF ANTICHOLINERGIC DRUGS ON HIDROSE SUBJECTS

Drug	Type of Hyperhidrosis: Mild Effective	Mild Ineffective	Pronounced Effective	Pronounced Ineffective	Severe Effective	Severe Ineffective	Total Percentage Effectiveness
Pro-Banthine	20	3	11	2	0	3	79.5
Antrenyl	14	3	16	2	1	1	83.8
Pamine	10	1	7	2	2	0	86.4
Darstine	9	1	5	2	0	1	77.8
Prantal[a]	20	3	12	4	1	2	78.6
Banthine[a]	27	2	13	0	0	2	90.9

[a] Figures obtained from previously reported studies (3, 4).

Ninety-four or approximately 81% of the subjects in the test groups claimed effective control of hyperhidrosis. Effective dosages varied in mild cases from 45–60 mg. daily for Pro-Banthine, 15–20 mg. for Antrenyl, 7.5–10 mg. for Pamine, and 150–200 mg. for Darstine. In pronounced cases daily doses of 90 mg. Pro-Banthine, 30 mg. Antrenyl, 15 mg. Pamine, and 300 mg. Darstine were necessary for effective control. In the more severe cases daily doses of 90–120 mg. of Pro-Banthine, 30–60 mg. of Antrenyl, 15–30 mg. of Pamine, and 300–600 mg. of Darstine were optimal.

Of the 22 subjects in whom the anticholinergic drug being tested was ineffective, only 9 were effectively controlled by the substitution of another anti cholinergic agent. Five of these were controlled by Banthine, 2 by Pamine, 1 by Antrenyl, and 1 by Pro-Banthine. Neither Darstine nor Prantal was effective in controlling the hyperhidrosis of any subject in this group.

Quantitative Estimation of Sweat.—Two hundred and sixteen quantitative determinations of sweat on seven different areas of the body were performed. Approximately 75% of the subjects used were hidrose. The procedure used was similar to that previously described (4). There was no significant difference between the response of normal and hidrose subjects to administration of the drugs. Table III shows the comparative effect of these drugs in reducing sweat on various parts of the body. From this table it will be noted that Pamine and Darstine reach a point of effectiveness within thirty minutes and a peak of effectiveness somewhere between thirty to sixty minutes. Pro-Banthine and Antrenyl reach a point of effectiveness between thirty to sixty minutes and a peak of effectiveness somewhere between sixty to ninety minutes. None of the drugs under study seemed to show further appreciable reduction of sweat beyond ninety minutes after drug administration. Darstine appeared to possess the greatest rapidity of action and Pamine the longest duration of action.

In four areas (dorsum of the hand, forearm, forehead, and thigh) the average reduction of sweat is greatest with Pamine under the experimental conditions stated. Pro-Banthine is the most effective in two areas (upper arm and leg) and Darstine in only one area, the dorsum of the foot. Antrenyl appears to be the second most effective agent in five of the seven body areas tested. No attempt is made to explain the apparent superiority of one drug over another on a specific body area,

TABLE III.—EFFECT OF PRO-BANTHINE, ANTRENYL, PAMINE, AND DARSTINE ON AVERAGE PERCENTAGE REDUCTION OF SWEAT IN NORMAL AND HIDROSE SUBJECTS

Area of Body	Pro-Banthine (30 Mg.) 30	60	90	Antrenyl (10 Mg.) 30	60	90	Pamine (5 Mg.) 30	60	90	Darstine (100 Mg.) 30	60	90
	Minutes After Ingestion											
Dorsum of hand	0.6	18.7	17.3	19.7	16.9	17.1	21.3	20.1	22.4	19.3	16.4	11.4
Forearm	2.3	19.4	19.9	13.4	26.0	23.8	29.7	22.3	24.7	21.1	18.3	17.6
Upper arm	1.1	21.8	22.3	11.6	20.4	17.6	19.1	18.4	17.3	18.4	19.2	11.8
Forehead	6.9	38.4	36.9	9.9	33.5	32.1	27.3	38.6	31.3	33.1	24.7	18.6
Dorsum of foot	0.9	27.3	28.1	11.0	31.6	27.0	21.9	23.4	20.8	31.0	32.3	22.9
Leg	3.4	28.2	26.7	6.6	14.3	15.6	18.0	26.3	20.1	16.3	11.9	15.7
Thigh	2.2	24.6	20.9	8.3	28.2	19.9	19.7	32.3	27.6	11.2	19.9	21.3

TABLE IV.—COMPARATIVE SIDE EFFECTS OF THE ANTICHOLINERGIC DRUGS

Effect	Percentage of Subjects Reporting Effects: Pro-Banthine	Antrenyl	Pamine	Darstine	Prantal[a]	Banthine[a]
Headache	10.2	21.4	7.7	9.1	8.0	27.1
Constipation	8.5	14.3	3.8	9.1	0.0	18.8
Diarrhea	0.0	0.0	0.0	0.0	6.0	0.0
Xerostomia	55.3	85.7	15.4	4.6	22.0	100.0
Mydriasis	4.2	4.7	3.8	4.6	4.0	6.3
Drowsiness	8.5	4.7	3.8	0.0	4.0	?
Gastric irritation	0.0	2.3	0.0	0.0	6.0	0.0
Dysuria	2.1	2.3	0.0	4.6	0.0	4.2
Heartburn	4.2	4.7	3.8	4.6	0.0	16.7
Xerophthalmia	2.1	2.3	0.0	0.0	0.0	0.0

[a] Figures obtained from previously reported studies (3, 4).

Under the same experimental conditions, quantitative determinations of sweat were performed on 18 hidrose and normal subjects who were administered placebos. Reduction of sweat ranged from 1.8 to 7.3% in 10 subjects but was increased from 2.1 to 6.3% in the other 8 subjects.

Side Effects.—Subjective manifestations of parasympathetic inhibition were reported with all the anticholinergic drugs under study. Table IV shows the side effects reported by the test subjects at the dosage level used for control of the hyperhidrosis by each subject. A comparison is also made of side effects reported in previous studies with Banthine and Prantal (3, 4). Side effects were reported by 9 controls and 37 hidrose subjects, several of whom reported more than one side effect. Most of the side effects were mild and of a transient character. In six instances the side effects became so pronounced that the subject was forced to discontinue the medication. Constipation and persistent headache necessitated discontinuance of Antrenyl in 4 of these subjects. Xerophthalmia with Pro-Banthine in one subject and dysuria with Darstine in another were of sufficient severity to warrant substitution of another anticholinergic drug. Under conditions of the present study Antrenyl apparently possesses the most pronounced side effects, followed by Pro-Banthine, Pamine, and Darstine in that order.

DISCUSSION

The effective control of hyperhidrosis without disturbing side effects is not possible with the anticholinergic drugs studied to date. Nevertheless continued progress is being made toward this objective but whether or not this will ever become a reality is doubtful.

Administration of the anticholinergic agents must be continued indefinitely for effective control of hyperhidrosis, as evidenced by the fact that when the drugs were discontinued the excessive sweat appeared with regularity. In our studies continued administration of the drugs resulted in the development of a tolerance toward only two of these agents, Banthine and Pro-Banthine.

Subjects with complaints of side effects were given placebos. In most cases relief from the side effects was reported. Several subjects claimed relief from hyperhidrosis with placebos, necessitating their being dropped from the test groups, since it was felt their subjective reports were unreliable. In general, the administration of placebos substantiated the subjective reports of the test groups. Further substantiation of reported side effects was obtained through the use of blind studies.

Under the experimental conditions stated, the order of maximum effectiveness in controlling hyperhidrosis was: Banthine, Pamine, Antrenyl, Pro-Banthine, Prantal, and Darstine. The most troublesome side effects were seen with Banthine, followed by Antrenyl, Pro-Banthine, Pamine, Darstine, and Prantal in that order. The correlation between suppression of sweating and incidence of side effects exists to some degree, although by no means is this relationship clear cut. It does appear, however, that anticholinergic agents with the greatest effectiveness in controlling hyperhidrosis also exhibit maximum side effects. A notable exception to this generalization is Pamine. The suppression of sweating may, therefore, be in direct ratio to the incidence of side effects to be anticipated in continued anticholinergic administration.

SUMMARY

1. One hundred and sixteen hidrose and 21 normal subjects were administered Pro-Banthine, Antrenyl, Pamine, and Darstine for periods ranging from six weeks to eight months. None of the anticholinergic drugs under study was completely effective in controlling hyperhidrosis. Thirteen hidrose subjects failed to respond to treatment with any of these drugs.

2. Subjective manifestations of parasympathetic inhibition were reported with all the anticholinergic drugs under study. The reported side effects were substantiated by placebo administration and blind studies.

3. Quantitative estimations of sweat on various parts of the body were performed before and after drug administration. Pamine and Antrenyl proved most effective in suppressing sweat. The dosage used in these quantitative experiments was twice the recommended single oral dose of each drug.

4. Pamine and Darstine become effective within thirty minutes after administration while Pro-Banthine and Antrenyl reach a point of effectiveness in thirty to sixty minutes. The peak of effectiveness is reached somewhere between thirty to sixty minutes with Pamine and Darstine and sixty to ninety minutes with Pro-Banthine and Antrenyl.

5. Studies to date indicate that the order of maximum effectiveness in controlling hyperhidrosis under the experimental conditions stated is: Banthine, Pamine, Antrenyl, Pro-Banthine, Prantal, and Darstine. The most troublesome side effects are seen with Banthine, followed by Antrenyl, Pro-Banthine, Pamine, Darstine, and Prantal.

6. It appears that the anticholinergic drug possessing maximum effectiveness in controlling hyperhidrosis also has a greater incidence of side effects. Pamine is a notable exception to this generalization.

7. Although the drugs effectively control hyperhidrosis, the administration of the anticholinergic drugs studied must be continued indefinitely.

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Spectrophotometric Determination of Morphine and of Codeine*

By WILLIAM A. CLARK† and ARTHUR J. McBAY‡

Morphine can be distinguished from codeine by the shift of the maximum absorption value of morphine in alkaline medium from that in acid medium. Two spectrophotometric methods, the absolute and the relative methods, are presented for the determination of morphine and of codeine. Equations are given for the calculation of the concentrations of alkaloids in unknown samples. The relative method required a standard each time a sample was assayed while the absolute method did not require a standard. Samples of morphine and codeine were assayed by each of these methods. The percentages of error ranged from 0.4 per cent to 2.46 per cent, using the relative method, and from 0.85 per cent to 2.34 per cent, using the absolute method. No significant difference in precision was found between the two methods.

ALTHOUGH morphine and codeine have been used for over one hundred years, no rapid and accurate method for their identification and assay has been advanced. In this paper rapid spectrophotometric methods of identification and of quantitative analysis are reported.

All of the assays of the U. S. P. and N. F. for morphine and codeine and the salts of these alkaloids have been extraction-titration type assays. These assays are time-consuming and they require a relatively large amount of sample to obtain a fair degree of accuracy of assay. These disadvantages indicate the need for a different type of assay for these alkaloids.

A capable survey of the official tests for identification of morphine and of codeine and their various official forms has appeared elsewhere (1). The tests are satisfactory for those who use them routinely but they leave much to be desired for those who only refer to them occasionally.

Since morphine and codeine are known to absorb ultraviolet light, spectrophotometry offers a chance not only for the quantitative analysis of these alkaloids, but also for their identification. There are several apparent advantages in the use of spectrophotometry. First, very little time is required to assay a sample since the assay consists merely of the dilution of the sample and the reading of the absorbance of this diluted sample. Second, the small amount of sample which is required is not destroyed and could be recovered if necessary. Third, if the assay is properly designed the alkaloid can be identified at the same time that it is being assayed. These advantages indicate the usefulness of spectrophotometry in obtaining precise and rapid methods of assay and identification which could entirely replace the present methods.

Henri and Gompel (2) applied ultraviolet absorption spectra in an attempt to determine the structure of codeine and morphine. They found that morphine and codeine had almost identical curves. Bontempi (3) reported that morphine and morphine hydrochloride had the same spectrum, and concluded that the hydrochloric acid had no effect upon the spectrum of morphine. Eisenbrand (4) reported that morphine could be determined in amounts as low as 0.2 mg. by means of absorption spectra. Elvidge (5) published absorption spectra for morphine and codeine and some of their salts. His results show that the spectra for morphine, codeine, and codeine phosphate are very similar, their minima being about 260 mμ and maxima about 280 mμ. The spectrum for morphine in sodium hydroxide solution differed from that in isopropyl alcohol. Csokan (6) published absorption spectra for morphine and codeine in water and alcohol. Ultraviolet spectrophotometric curves of morphine at various *p*H values appeared after this work was completed (7).

EXPERIMENTAL

Ultraviolet Absorption Studies.—All ultraviolet measurements were made over the range from 220 to 325 mμ, on a Beckman DU spectrophotometer in 1-cm. cuvettes using a slit width of 0.5 mm. unless otherwise indicated. All solutions, whether prepared from the alkaloid or the sulfate, were calculated to contain the indicated amount of anhydrous alkaloid base. A Beckman Model H *p*H meter was used to determine the *p*H.

Since these alkaloids usually contain moisture as well as water of crystallization, it was necessary to determine the exact amount present in the samples. This was done by means of the Karl Fisher Reagent (8).

Method of Identification.—Since both morphine and codeine have absorption maxima and minima at

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† Abstracted from a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by William A. Clark in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

‡ Associate Professor of Chemistry, Massachusetts College of Pharmacy, Boston.

TABLE I.—RELATIONSHIP BETWEEN ABSORBANCE AND *p*H

	Morphine (100 Mcg./Cc.)				Codeine (100 Mcg./Cc.)			
	Min.		Max.		Min.		Max.	
*p*H	Mμ	Abs.	Mμ	Abs.	Mμ	Abs.	Mμ	Abs.
2	262	0.167	286	0.531	262	0.184	284	0.538
4	262	0.164	286	0.529	262	0.184	284	0.538
6	262	0.167	286	0.529	262	0.188	284	0.533
8	264	0.327	286	0.546	262	0.155	284	0.499
10	278	0.535	294	0.689	262	0.153	284	0.476
12	280	0.545	298	0.892	262	0.187	284	0.497

approximately the same places in acid media (Table I), it was not possible to distinguish one from the other in acid media. However, in strongly alkaline media, morphine has an absorption minimum at approximately the same wave length that codeine has an absorption maximum. This phenomenon made it possible to distinguish morphine from codeine. (See Figs. 1 and 2.)

This method of identification is valid only if the substance to be identified is suspected to be either morphine or codeine. Many substances, particularly other alkaloids of opium, may possess the same maxima and minima as do morphine and codeine in alkaline medium.

If the absorbance at 285 mμ is greater than that at 310 mμ, the substance is codeine and if the absorbance at 310 mμ is greater than that at 285 mμ, the substance is morphine. The ratio of the maximum to the minimum absorbance could serve as an official index of purity for either of these alkaloids.

Quantitative Analysis.—Data are presented for the determination of the concentration of morphine and of codeine at both *p*H 2 and *p*H 12. Although morphine has a greater absorbance at 298 mμ and a *p*H of 12, there are times when it would be more advantageous to work with solutions on the acid side which are not as sensitive to changes of *p*H. Mixtures of morphine and codeine may be analyzed and the amounts of each of the alkaloids may be determined simultaneously by using two-component system equations. This work will be presented in a later paper.

Absolute Method.—Tables II and III present data which were used to establish equations for determining the concentration of the alkaloids by the absolute method.

TABLE II.—RELATIONSHIP BETWEEN CONCENTRATION AND ABSORBANCE FOR MORPHINE

*p*H of 2,285 Millimicrons			*p*H of 12,298 Millimicrons		
Conc., Mcg./Cc.	Abs.	Conc./ Abs.	Conc., Mcg./Cc.	Abs.	Conc. /Abs.
62.23	0.326	190.9	35.56	0.329	108.1
64.72	0.339	190.9	36.98	0.342	108.1
67.31	0.353	190.8	38.46	0.353	109.0
70.00	0.363	192.8	40.00	0.362	110.5
	Av.	191.3		Av.	108.9

Relative Method.—The relative method (9) consisted essentially of measuring the absorbance of an unknown sample of morphine or codeine against a sample of known concentration of alkaloid.

A preliminary experiment was carried out to see if this method could be applied to solutions of morphine and of codeine. The following procedure was adopted:

TABLE III.—RELATIONSHIP BETWEEN CONCENTRATION AND ABSORBANCE FOR CODEINE

*p*H of 2,285 Millimicrons			*p*H of 12,284 Millimicrons		
Conc., Mcg./Cc.	Abs.	Conc./ Abs.	Conc., Mcg./Cc.	Abs.	Conc./ Abs.
62.23	0.332	187.4	62.23	0.303	205.4
64.72	0.350	184.9	64.72	0.315	205.5
67.31	0.360	187.0	67.31	0.323	208.4
70.00	0.374	187.2	70.00	0.335	208.9
	Av.	186.6		Av.	207.1

(*a*) A stock solution of morphine was prepared which contained 150 μg./cc., with 0.02 *N* sodium hydroxide as the solvent.

(*b*) One liter, accurately measured, was taken and exactly 10 cc. of 0.02 *N* sodium hydroxide was added and this solution was thoroughly mixed. A sample of this dilution was taken.

(*c*) The remainder of the above solution was diluted to contain 125 μg./cc. of morphine and a similar one-hundredth dilution was prepared.

(*d*) This procedure of dilution was continued in the same manner until six pairs of samples were obtained containing 150, 125, 100, 75, 50, 25 μg./cc. and a one one-hundredth dilution of each of these concentrations. The absorbance of each concentration and the dilution was determined with 0.02 *N* sodium hydroxide as a blank and also with water as a blank. Then, using the more dilute of the pair of solutions as a blank, the transmittance of the more concentrated solution was obtained. From these absorbances and transmittances, the concentration ratios (α) were calculated from the following formula:

$$\log (I_1/I_2) = A_1 (\alpha - 1)$$

where (I_2/I_1) equals the transmittance of the more concentrated solution, measured against the more dilute solution and A_1 equals the absorbance of the more dilute solution measured against the 0.02 *N* sodium hydroxide. Table IV shows all values obtained and the calculated concentration ratios. The expected concentration ratios were 1.01. The calculated ratios had an average deviation of 0.006 from the expected ratios. This preliminary experiment failed to show an advantage in the use of the relative method of analysis for alkaloids such as morphine and codeine over the absolute method. Since this work was completed, Hiskey (10) found that his method was less precise than he first reported (9).

Equation for the Calibration Curve.—A solution of codeine containing 70 μg./cc. of codeine was prepared using 0.02 *N* sulfuric acid as the solvent. Two-hundred and fifty cubic centimeters was accurately measured. Exactly 10 cc. of 0.02 *N* sulfuric acid was added and the solution was thoroughly shaken; 10 cc. of this solution was withdrawn as a sample.

TABLE IV.—PRECISION OBTAINED WITH MORPHINE SYSTEM

Conc. Mcg./Cc	A (Dilute vs. NaOH)	A_1 (Conc. vs. NaOH)	Absolute α	Absolute Dev.	I_2/I_1	Relative α	Relative Dev.
150	1.358	1.372	1.0103	0.0003	0.976	1.0078	0.0022
125	1.082	1.095	1.0120	0.0020	0.973	1.0109	0.0009
100	0.895	0.900	1.0056	0.0044	0.989	1.0054	0.0046
75	0.639	0.649	1.0156	0.0056	0.978	1.0150	0.0050
50	0.446	0.450	1.0090	0.0010	0.991	1.0088	0.0012
25	0.207	0.214	1.0338	0.0238	0.984	1.0338	0.0238
Av. deviation				0.0062			0.0063

Another 10-cc. portion of 0.02 N sulfuric acid was added and the solution was thoroughly shaken. Ten cubic centimeters of this dilution was withdrawn. This process was repeated until three dilutions of the original solution had been obtained. At a wave length of 285 mμ the absorbance of each of these samples was obtained with 0.02 N sulfuric acid as a blank and then the absorbances of the three samples were again obtained, using the sample lowest in concentration of codeine as a blank. From these transmittance readings, the equation for the calibration curve was calculated (Table V). The equation for the calibration curve of codeine at a wave length of 285 mμ follows:

$$\text{Conc. } (\mu g./cc.) \text{ of codeine} = 169\ A_1\ (\alpha - 1) + 62.23$$

where $A_1\ (\alpha - 1)$ is the difference in absorbance between the standard (62.23 μg./cc.) and the solution compared with it.

The same procedure was carried out on morphine, using an original solution which contained 70 μg./cc. of morphine. The wave length used was 285 mμ (Table V).

The equation for the calibration curve of morphine follows:

$$\text{Conc. } (\mu g./cc.) \text{ of morphine} = 198\ A_1\ (\alpha - 1) + 62.23$$

These equations may be used for any concentration of morphine or of codeine by making the value added to the equation equal to the concentration of the standard used in the assay.

To prove the value of the proposed methods of assay, six samples of morphine and six samples of codeine were weighed out. Each of these samples was dissolved in 0.02 N sulfuric acid. The standard solutions of morphine and of codeine, prepared for the relative method, each contained 70 μg./cc. of alkaloid.

The calculated amounts of alkaloid, the amounts found by both the relative and absolute method, and the per cent of error of each method is given for morphine and codeine in Table VI.

DISCUSSION

The investigation of alkaloidal materials such as morphine and codeine is always hampered by the unavailability of the pure chemical or, in this case, the reference material. It would be of advantage if official material were made available for comparative assays, or if the official standards were adequate enough for one to be sure of the material that he is using. An extinction coefficient, or a ratio between the maximum and minimum absorbances, would be most helpful in establishing the purity of these chemicals.

Although morphine and codeine may be distinguished from one another and from a number of other materials by preparing spectrograms of the materials over several pH ranges, it was felt that the simple and shorter method presented in this paper would be adequate and would overcome the objections presented to the color tests (1).

The relative and the absolute methods of assay are studied, but since there seems to be no increase in accuracy and precision to an assay by the relative method over the absolute method and since it is necessary to prepare a standard for comparison for the relative method, the absolute method is pre-

TABLE V.—DATA FOR THE RELATIVE METHOD

Conc., Mcg/Cc.	Δc	Relative Transmittances 1	2	3	4	5	Av.	Av. Dev.	$A_1(\alpha - 1)$	$\frac{\Delta c}{A_1(\alpha - 1)}$
					Codeine					
62.23	..	1.000	1.000	1.000	1.000	1.000	1.000	...	...	...
64.72	2.49	0.964	0.965	0.964	0.965	0.965	0.965	0.0008	0.0155	160.65
67.31	5.08	0.937	0.936	0.937	0.937	0.938	0.937	0.0008	0.0283	179.51
70.00	7.77	0.898	0.899	0.899	0.898	0.899	0.899	0.0012	0.0462	168.18
									Av.	169.45
									Av. Dev	6.71
					Morphine					
62.23	..	1.000	1.000	1.000	1.000	1.000	1.000	...	...	...
64.72	2.49	0.971	0.970	0.975	0.971	0.972	0.972	0.0014	0.0123	202.44
67.31	5.08	0.939	0.941	0.941	0.941	0.941	0.941	0.0004	0.0264	192.43
70.00	7.77	0.913	0.915	0.914	0.915	0.914	0.914	0.0008	0.0391	198.72
									Av.	197.86
									Av. Dev.	3.62

TABLE VI.—SAMPLES ASSAYED BY RELATIVE AND ABSOLUTE METHODS

Absorbance			Conc., Mcg./Cc.			Per Cent Error	
Stand.	Sample	$A_1(a-1)$	Calc.	Rel.	Abs.	Rel.	Abs.
			Morphine				
0.363	0.371	0.0074	71.8	71.5	71.0	0.42	1.11
0.362	0.387	0.0227	74.8	74.5	74.0	0.40	1.07
0.361	0.368	0.0052	71.6	71.0	70.4	0.84	1.68
0.363	0.375	0.0119	73.0	72.4	71.7	0.82	1.78
0.362	0.373	0.0092	72.2	71.8	71.4	0.55	1.11
0.362	0.367	0.0044	71.4	70.9	70.2	0.70	1.68
					Av.	0.62	1.40
			Codeine				
0.374	0.376	0.0013	71.2	70.2	70.1	1.40	1.54
0.374	0.384	0.0083	73.2	71.4	71.6	2.46	2.17
0.374	0.374	0.0000	70.6	70.0	70.0	0.85	0.85
0.374	0.382	0.0083	72.8	71.4	71.1	1.92	2.34
0.374	0.380	0.0048	72.0	70.8	70.9	1.67	1.53
0.374	0.383	0.0070	72.6	71.2	71.4	1.93	1.65
					Av.	1.70	1.68

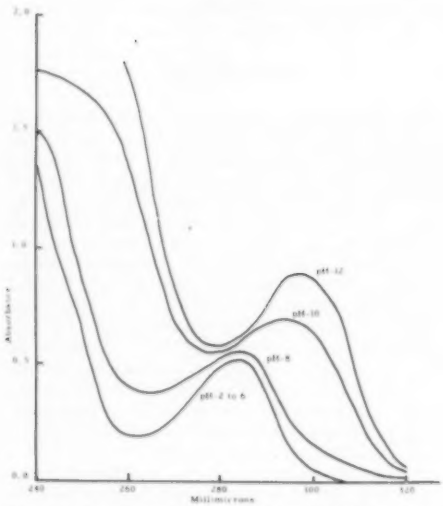

Fig. 1.—Spectrogram of morphine (100 Mcg./cc.)

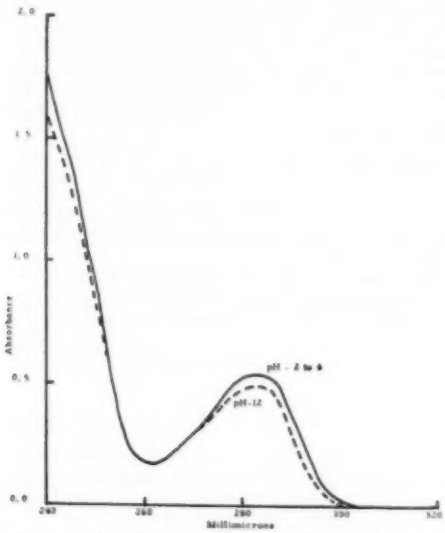

Fig. 2.—Spectrogram of codeine (100 Mcg./cc.)

ferred. An alternative to preparing a fresh standard for the relative method would be to prepare a stable solution of some other ultraviolet absorbing material for use as a comparison standard.

SUMMARY

1. A simple and rapid method of assay for morphine and for codeine has been developed and evaluated.

2. Morphine and codeine may be distinguished by a simple and positive method.

3. The absolute method of assay is preferred over the relative method for morphine and codeine.

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A Preliminary Phytochemical Study of *Hippomane mancinella* L.*

By H. J. SCHAEFFER, W. M. LAUTER,† and P. A. FOOTE‡

A phytochemical study was made on *Hippomane mancinella* L. Alpha-carotene and 2-hydroxy-4,6-dimethoxyacetophenone were isolated from the leaves. The most toxic extract was the aqueous extract of the wood and the alcoholic extract was the next most toxic; however, the toxic constituents were not isolated.

SINCE 1850, *Hippomane mancinella* L. has been classified as one of the most poisonous trees known, yet few phytochemical studies have been made for its toxic principles. It was the purpose of this research to attempt to isolate both toxic and nontoxic substances and to identify them. In plant analysis decomposition occurs so easily that the isolated nontoxic materials may be decomposition products of the toxic substances; hence, they may give a clue to the structure of the toxic substances. Lauter, Fox, and Ariail (1) have previously reviewed the literature.

Both the leaves and the wood of *Hippomane mancinella* L. were examined in this phytochemical study. The procedures used consisted of steam distillation of the leaves and the wood, extraction of the leaves with distilled water and with acidified ethanol, and extraction of the wood with acidified water, 95 per cent ethanol, and chloroform.

The extracts were examined by extraction with immiscible solvents and by column chromatography. Special procedures of analysis were employed wherever indicated.

EXPERIMENTAL

Source of Material and Its Storage.—Two trips were made to the Everglades National Park, one in August, 1951, and one in December, 1951, to collect the material to be examined. Dan Baird, Superintendent, and his assistants identified the tree and gave us permission to remove samples of the wood, leaves, and the fruit. The material was stored at 0° F. until used.

Moisture Content, Total Ash, and Acid-Insoluble Ash of the Leaves and Wood.—The procedures described in the U. S. P. XIV (2) were followed. The results are listed in Table I.

TABLE I.—ANALYSIS OF THE LEAVES AND WOOD OF HIPPOMANE MANCINELLA L.

	Leaves, %	Wood, %
Moisture	62.2	38.2
Total ash	10.6	0.10
Acid-insoluble ash	3.16	0.09

Steam Distillation of the Leaves and Isolation of 2-Hydroxy-4,6-dimethoxyacetophenone.—Four and one-half kilograms of fresh leaves were ground to a fine powder in a corn grinder. This powder was steam distilled until 24 L. of distillate had been collected. Soon after distillation began a white crystalline compound precipitated. The distillate was placed in a refrigerated room (4° C.) for twenty-four hours and then it was filtered. The yield of the crude compound was 3.89 Gm. or 0.09% of the fresh leaves. It was recrystallized from 50% ethanol and melted at 83.5°. This compound was identified as 2-hydroxy-4,6-dimethoxyacetophenone by means of its bromo derivative and its oxime, the melting points of which check with those found in the literature (3). A 2,4-dinitrophenylhydrazone was prepared in a 60% yield. Its melting point was 230–231°. The ultraviolet absorption spectrum of 2-hydroxy-4,6-dimethoxyacetophenone was determined in 95% ethanol on a Beckman D. U. quartz spectrophotometer using a slit width of 0.75 mm. The absorption spectrum is shown in Fig. 1.

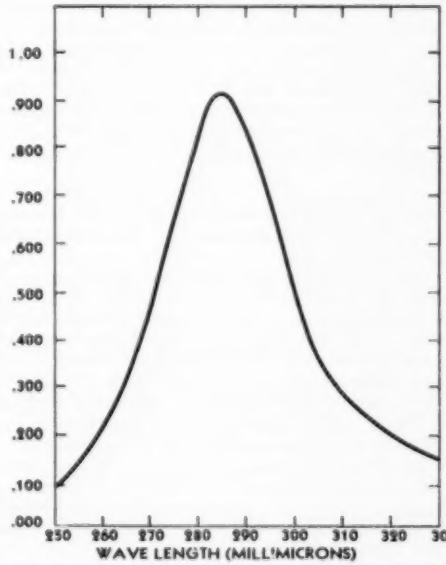

Fig. 1.—Ultraviolet absorption spectrum of 2-hydroxy-4,6-dimethoxyacetophenone.

* Received July 20, 1953, from the College of Pharmacy, University of Florida, Gainesville.

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† Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Florida.

‡ Dean, College of Pharmacy, University of Florida.

Anal.—Calcd. for $C_{10}H_{12}O_4$: C, 61.26; H, 6.17. Found: C, 61.38; H, 6.30.

This compound has previously been isolated from *Artemisia brevifolia* Wallich by Smith and Smith (4) in 1927. They gave the name Brevifolin to 2-hydroxy-4,6-dimethoxyacetophenone.

Preparation and Examination of the Aqueous Extract of the Leaves.—After steam distillation of the leaves was completed, there remained a considerable portion of water in the flask. This aqueous portion was separated from the leaves by filtration and was considered to be a good aqueous extract. The extract was cooled to 4° for seventy-two hours, but no change was noted at the end of this period. The extract was then concentrated by vacuum distillation at a temperature less than 35° to a volume of 1 L. This concentrated extract had a deep brown color and could not be decolorized with animal charcoal.

Five cubic centimeters of this extract was made acid with diluted hydrochloric acid and it was extracted four times with 10-cc. portions of petroleum ether, benzene, ether, and chloroform, in the order mentioned. It was then made alkaline with ammonia test solution and extracted in the same manner and with the same solvents as was the acid solution. Each extract was evaporated to dryness on a steam bath. No crystalline products were obtained by this procedure; however, a small amount of a reddish-yellow wax was obtained from the ether extract of the acid solution.

Fifty cubic centimeters of the remaining concentrated extract was precipitated with 95% ethanol. The solution was cooled for twenty-four hours and then filtered. The filter cake was redissolved in hot water and 95% ethanol was added until a faint cloudiness occurred. This solution was placed in a refrigerator for twenty-four hours but no precipitate was noticed.

Preparation and Examination of the Alcoholic Extract of the Leaves.—One thousand and thirty grams of the dried leaves which had been previously steam distilled was extracted by percolation using 95% ethanol, containing 0.05 cc. of concentrated hydrochloric acid per 100 cc. of ethanol. The percolate was concentrated to 450 cc. by vacuum distillation at a temperature less than 35°. This was cooled for ninety-six hours but no change was noted.

One hundred cubic centimeters of the concentrated alcoholic extract was extracted with 50-cc. portions of petroleum ether until the petroleum ether layer was colorless.

The alcoholic extract which had been shaken out with petroleum ether was examined by column chromatography, using activated alumina grade F-20 as the adsorbent. The column was developed with 95% ethanol. One yellow band formed which was removed from the column; however, insufficient material was obtained to allow further work.

The petroleum ether extract of the alcoholic extract of the leaves was examined by column chromatography using calcium phosphate as the adsorbent. The column was 2.3 cm. in diameter and 25 cm. in length. Forty cubic centimeters of the petroleum ether extract was added to the column and it was developed with petroleum ether. A total of nine bands was formed. The bands are described below in numerical terms, that is, the narrowest band is given the numeral 1 and the width of every other band including the colorless zones between the bands are described as multiples of the narrowest band.

Light green	2
Dark green	4
Light green	1
Colorless	8
Light yellow	1
Pink	1
Colorless	1
Light yellow	1
Colorless	21
Very light pink	1
Colorless	3
Yellow	19
Colorless	18

The adsorbent could not be extruded from the column, so the glass tube was cut in order to remove the bands. The lowest yellow band was removed in a pure form but the remaining bands were so close to each other that they could not be removed as pure bands.

The adsorbent containing the lowest yellow band was extracted with ether and filtered. The filtrate was evaporated to dryness on a steam bath. A waxy reddish-yellow material was obtained. A small portion of this material was redissolved in ether, and concentrated sulfuric acid was added so that two layers were formed. A blue color formed at the interface of the ether and the concentrated sulfuric acid, suggesting a carotenoid substance.

The adsorption spectrum of this material was determined on a Beckman D. U. quartz spectrophotometer, using carbon disulfide as the solvent. The absorption spectrum was typical of α-carotene with maximum absorption at 477 and 509 mμ.

Steam Distillation of the Wood.—One hundred grams of the ground wood was steam distilled until 500 cc. of distillate was collected. The distillate was cooled for twenty-four hours but no oil or solid separated.

Five cubic centimeters of the distillate was evaporated to dryness on a watch glass and no residue was obtained.

The distillate was tested by the pharmacology department and was found to be free from toxic substances (6).

Preparation and Examination of the Aqueous Extract of the Wood.—Five and four-fifths Kg. of the ground wood was extracted by percolation with acidified water (0.05% HCl) until a total of 21 L. had been collected; 50 cc. of this extract was shaken four times with 10-cc. portions of petroleum ether, benzene, ether, and chloroform. Then the extract was made alkaline with ammonia test solution and was extracted in the same manner as was the acid solution. Each extract was evaporated to dryness on a steam bath but no products were isolated by this procedure.

Five hundred cubic centimeters of the aqueous extract was decolorized with animal charcoal. Samples of the original and the decolorized extracts were found to be equally toxic when pharmacologically tested (6). It was found that after evaporating the extract to dryness on a steam bath, the toxicity was decreased, indicating thermolability of the toxic constituents (6).

The decolorized extract did not react with Mayer's reagent, picric acid solution, or ferric chloride test solution. It slowly discharged the colors of bromine water and potassium permanganate test solutions. It gave a positive carbohydrate test and reacted with Benedict's solution.

Column chromatography was employed using calcium phosphate as the adsorbent, but this method of analysis was unsuccessful.

The aqueous extract produced a yellow precipitate when it was treated with an alcoholic solution of nitranilic acid. The procedure described by Schmall, Wollish, and Galender (5) was employed to prepare the nitranilate compound with the exception that 1 L. of the extract was decolorized and concentrated to a volume of 100 cc. *in vacuo*, and an equal volume of the nitranilic acid solution was added to it, rather than using a weighed quantity of material. The yield of the nitranilate compound was 2.48 Gm. It was insoluble in water and all common organic solvents and it did not melt below 300°. It was found in the combustion analysis that the compound was explosive. An attempt was made to hydrolyze the compound with 40% sodium hydroxide solution and with concentrated hydrochloric acid, but no identifiable products were obtained.

Anal.—Found: C, (Wet Combustion), 23.69%; N, (Kjeldahl Method), 9.14%.

Because of excessive foaming of the aqueous extract it was thought that the toxicity may be due to a sapotoxin; therefore, 4.8 Kg. of fresh wood was extracted by percolation with distilled water until a total of 25 L. was collected. This was decolorized with animal charcoal and concentrated to a volume of 2 L. by vacuum distillation. To this solution was added 2 L. of a saturated aqueous solution of barium hydroxide slowly and with stirring. It was allowed to stand for four hours and then filtered. A tan flocculent precipitate was obtained which was resuspended in 1 L. of 0.01 *N* sulfuric acid. Carbon dioxide was bubbled into the solution for six hours. It was then filtered and concentrated by vacuum distillation to a volume of 200 cc. A sample of this concentrated extract was pharmacologically evaluated and was found to be toxic (6); therefore, 50 cc. of this concentrated solution was further concentrated by vacuum distillation to a volume of 10 cc. and placed in a refrigerator, but no material crystallized from this solution.

Preparation and Examination of the Alcoholic Extract of the Wood.—One kilogram of the dried wood, which had been previously extracted with water, was extracted by percolation using 95% ethanol. The percolate was concentrated to a volume of 500 cc. by vacuum distillation at a temperature less than 34°.

Thirty cubic centimeters of the alcoholic extract was extracted with successive portions of petroleum ether until they became colorless. The petroleum ether was allowed to evaporate to dryness and a negligible residue was obtained. The alcoholic extract which had been shaken out with petroleum ether was concentrated to a volume of 20 cc. and placed in a refrigerator for twenty-four hours, after which no change was noted.

Attempts to examine the alcoholic extract by means of column chromatography using activated alumina grade F-20 as the adsorbent were not successful and no well-defined products were obtained.

Preparation and Examination of the Chloroform Extract of the Wood.—Two hundred and eighty grams of dried wood which had been previously extracted with water and with 95% ethanol was percolated with chloroform until the percolate was colorless.

Fifty cubic centimeters of this extract was extracted four times with 10-cc. portions of distilled water. The aqueous extract was evaporated to dryness and no residue was obtained.

The remaining chloroform extract was evaporated to dryness and a waxy residue was obtained weighing 0.49 Gm. It could not be crystallized from ethanol. A sample of this wax was pharmacologically tested and was found to be free from toxic reactions (6); therefore, work was not continued on this extract.

SUMMARY

1. A preliminary phytochemical study was made on *Hippomane mancinella* L. The moisture contents, the per cent total ash, and the per cent acid-insoluble ash of the leaves and the wood were determined.

2. Both the leaves and the wood were steam distilled. The preparation and the examination of the aqueous and alcoholic extracts of the leaves and wood are described. The examination of the chloroform extract of the wood is reported.

3. Brevifolin or 2-hydroxy-4,6-dimethoxyacetophenone was isolated from the steam distillate of the leaves and identified. The following derivatives of it were made: the bromo, the oxime, and the 2,4-dinitrophenylhydrazone. The compound was found to be absent from the wood.

4. α-Carotene was isolated from the alcoholic extract of the leaves. It was identified by its absorption spectrum determined on a Beckman D. U. quartz spectrophotometer.

5. The aqueous extract of the wood was found to be the most toxic one prepared and the alcoholic extract of the wood appeared to be the next most toxic; however, the toxic constituents were not isolated.

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Microtoxicology. X*

Colorimetric Reactions and Optical Crystallographic Properties of Five Synthetic Antispasmodics

By THOMAS J. HALEY† and GEORGE L. KEENAN‡

The antispasmodics Profenil Citrate, Syntropan phosphate, Trasentin hydrochloride, Pavatrine hydrochloride, and Lachesine chloride were subjected to a large number of colorimetric and precipitation reagents. Although definite differences between these drugs were observed, the drugs were difficult to identify by these methods. On the other hand, their optical crystallographic properties enable a positive identification to be made from very small amounts of material. Combination of these methods with melting point determinations may be useful to the toxicological chemist.

THE MANY undesirable side reactions and the great toxicity of the belladonna alkaloids have resulted in a search for new synthetic compounds with decreased toxicity and more selective antispasmodic activity. Among these new compounds are Profenil citrate (bis-γ-phenylpropylethylamine citrate), Syntropan phosphate (*dl*-tropic acid ester of 3-diethylamino-2,2-dimethyl-1-propanol phosphate), Trasentin hydrochloride (diphenylacetic acid ester of diethylaminoethanol hydrochloride), Pavatrine hydrochloride (diethylaminoethyl-7-fluorene carboxylate hydrochloride) and Lachesine chloride (benzilic acid ester of hydroxyethyl-dimethylethyl ammonium chloride).

Inasmuch as toxicity may be encountered from the use of these compounds and because little is known concerning either their microchemical reactions or their optical crystallographic properties, we have investigated them with the usual microtoxicological methods.

EXPERIMENTAL

The uncorrected melting points of the drugs given in Table I were determined with a Melt-Pointer electrically heated apparatus fitted with capillary tubes and Anschutz thermometers.

The reagents were prepared as directed by Thienes and Haley (1) or according to the reference listed in Tables II and III. The compounds were tested by placing one drop of reagent on a microscope slide or in the well of a spot-plate and adding about 0.1 mg. of the drug to it. The results of this addition were observed for approximately thirty minutes and the changes which occurred were recorded. In the case of the acidic colorimetric reagents, the end point was the formation of a stable color or charring of the drug, but all colors which appeared prior to this were used for identifying purposes. Tables II and III list the results obtained with the colorimetric and precipitation procedures. As a further aid in identifying and differentiating these antispasmodics, the optical crystallographic properties of the pure compounds recrystallized from ethanol-ether were studied by the immersion method. Table IV lists the optical crystallographic properties.

RESULTS

Profenil Citrate.—This consists of large amorphous lumps unsuitable for optical crystallographic study.

Syntropan Phosphate.—This consists of colorless tetragonal prisms of the first order. Under parallel polarized light the crystals extinguish sharply. Dark-field illumination shows the tetragonal prisms better than ordinary light. In convergent polarized light, uniaxial interference figures are rarely shown.

Transentin Hydrochloride.—This consists of small colorless rods. Under parallel polarized light these rods extinguish sharply. In convergent polarized light, the rods extinguish sharply and only flash interference figures are observed. Although the α and γ indices are shown crosswise on the rods, they are uncommon. The β index is most commonly found. Many rods oriented with their long axis perpendicular to the vibration plane of the lower Nicol prism will show orange and blue borders when mounted in liquid with $n = 1.612$.

TABLE I.—MELTING POINTS OF FIVE SYNTHETIC ANTISPASMODICS

Drug	Melting Point ° C. Found	Melting Point ° C. Recorded	Reference
Profenil citrate	100–101.5	.....	..
Syntropan phosphate	137–139	142–145	2
Trasentin hydrochloride	110.5–112.5	113–114	3
Pavatrine hydrochloride	140–141	143–144	4
Lachesine chloride	201–202 dec	213	5

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† Chief of the Division of Pharmacology and Toxicology, Atomic Energy Project and Associate Clinical Professor of Industrial Medicine, School of Medicine, University of California at Los Angeles.
‡ Formerly Microanalyst, Food and Drug Administration. Deceased.

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TABLE II.—COLORIMETRIC REACTIONS OF SYNTHETIC ANTISPASMODICS

Reagent and Reference	Profenil Citrate	Syntropan Phosphate	Trasentin Hydrochloride	Pavatrine Hydrochloride	Lachesine Chloride
Conc. H_2SO_4 (1)	Colorless	Colorless	Colorless	Burnt orange	Bright orange changing to pink
Conc. HNO_3 (1)	Colorless	Colorless	Colorless	Faint yellow	Colorless
Mandelin (1)	Colorless	Colorless	Pale yellow	Pale yellow	Yellow
Marquis (1)	Reddish brown	Faint brown	Brown	Emerald green	Orange rapidly changing to emerald green
Buckingham (1)	Colorless	Colorless	Colorless	Black	Colorless
Frohde (1)	Colorless	Colorless	Colorless	Chocolate brown	Bright orange
Vitali (1)	Yellow	Violet	Violet	Orange brown rapidly turn-black	Green

Pavatrine Hydrochloride.—This consists of very small colorless needles. Under convergent polarized light, the needles are too small to show interference figures. All of the indices are easily found, the α lengthwise and the β and γ crosswise.

Lachesine Chloride.—This consists of short prismatic rods. Under parallel polarized light, brilliant interference colors are observed. In convergent polarized light, the majority of the rods extinguish sharply so that interference figures suitable for orientation would not be expected. On rods showing the maximum double refraction, two refractive indices commonly occur and may be considered respectively the α and β for the substance.

DISCUSSION

The differences in melting points in Table I may be related to the methods used in obtaining them. The controlled heating of the Melt-Pointer apparatus and the Anschutz thermometers could possibly give the more accurate results, but others may feel that the standardized method of the *U. S. Pharmacopeia* is more accurate.

The colorimetric reactions are not conclusive in themselves but they do assist in differentiating the five antispasmodics from one another, and by comparison with the tables in Thienes and Haley (1), from other alkaloids and synthetic compounds. Pavatrine and Lachesine were the most reactive compounds tested. Vitalis test gave the greatest differentiation between the compounds themselves and atropine. However, a combination of the colorimetric and precipitation reactions will give a greater assurance of identification of any particular compound.

The differences in reactivity of the compounds with the precipitation reagents is such that in general they can easily be identified with the reagents

TABLE III.—PRECIPITATION REACTIONS OF SYNTHETIC ANTISPASMODICS

Reagent and Reference	Profenil Citrate	Syntropan Phosphate	Trasentin Hydrochloride	Pavatrine Hydrochloride	Lachesine Chloride
Chloroauric acid (1)	Fine needles in feather-like bundles	Fine amorphous granules	Small rods	Small rods	Amorphous granules
Chloroplatinic acid (1)	Amorphous granules	Amorphous granules	Amorphous granules	Amorphous granules	Small tablets and rectangles
Satd. aq. soln. picric acid (1)	Amorphous granules	Flat rhomboidal plates	Feather-like large rods	Feather-like large rods	Triangular flat plates
Wagner (1)	Amorphous granules	Hexagonal plates with ribs to angles of the hexagons	Rectangular flat plates	Amorphous granules	Amorphous granules
Mayer (1)	Amorphous granules	Long needles	Flat plates	Amorphous granules	Amorphous granules
Marme (1)	Amorphous granules	Fine needles	Amorphous granules	Amorphous	Fine feather-like rods
Scheibler (6)	Fine needles arranged in spherulites	Amorphous granules	Amorphous granules	Amorphous granules	Amorphous granules
Potassium chlorate 10%	Amorphous granules	Small dentrites and crosses	Small flat rhomboidal plates	Amorphous granules	Large irregular flat plates, small dendrites and crosses
Potassium chromate 5%	Small red granules and large green dendrites	Green needles, rectangular plates and rods	Green amorphous granules	Green amorphous granules	Small green plates and rods

TABLE IV.—OPTICAL CRYSTALLOGRAPHIC PROPERTIES OF SEVERAL ANTISPASMODICS

Substance	Habit	Extinction	Elongation	Optic Sign	Refractive Indices[a]		
					Alpha	Beta	Gamma
					ω	ε	
Syntropan phosphate	Prisms	...	...	−	1.562	1.519	...
Trasentin hydrochloride	Small rods	Parallel	±	+	1.603	1.612	1.631
Pavatrine hydrochloride	Small needles	Parallel	−	...	1.583	1.670	1.690
Lachesine chloride	Prismatic rods	Inclined	...	...	1.575	...	1.638

[a] All values ±0.002.

used. Unless, however, the colorimetric reagents are used in conjunction with the precipitation ones, difficulty may be encountered in differentiating Trasentin from Pavatrine.

The optical crystallographic properties of these compounds are the best means for their positive identification and, when used in conjunction with the other tests, enable a rapid positive identification to be made with a minimum of time and material.

SUMMARY

The melting points, reactivity to colorimetric and precipitation reagents, and the optical crystallographic properties of five new synthetic antispasmodics have been investigated. It has been shown that these compounds can be differentiated from one another by the procedures used. Their optical crystallographic properties offer the best means for their positive identification.

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The Infrared Determination of Erythromycin*

By WILLIAM H. WASHBURN†

An infrared method for the analysis of erythromycin is described. A base line determination was developed employing the absorption at 10.46 μ, which was found to agree closely with the biological activity of the antibiotic. The method is simple and rapid to perform. Reproducibility is within ±1%.

THE RECENT INTRODUCTION of the antibiotic erythromycin,[1] represents an important contribution into the field of chemotherapy. This paper describes a rapid and precise method of determining the potency of erythromycin.

* Received June 11, 1953, from the Control Department, Abbott Laboratories, North Chicago, Ill.
† The author wishes to acknowledge the helpful suggestions of Dr. William B. Brownell, Head of Analytical Research, Control Department.
[1] This product is manufactured by Abbott Laboratories under the trade name Erythrocin.

EXPERIMENTAL

A preliminary qualitative spectrum was recorded from 7–12 μ, using a concentration of 30 mg./cc. in chloroform and a cell of 0.3 mm. thickness. A per cent transmittance curve (Fig. 1) was constructed from the single beam record. The absorption band at 10.46 μ was selected for a base line determination. Chloroform A. R. was used throughout.

Absorption measurements were made on solutions of erythromycin having concentrations in the range of 20–30 mg./cc., and a working curve was constructed plotting absorbancy versus concentration. A linear relationship was found over the range studied.

The purity and potency of the standard used in preparing this working curve were established by X-ray diffraction, melting point, solubility analysis, ultraviolet spectrophotometric assay, and micro, biological assay.

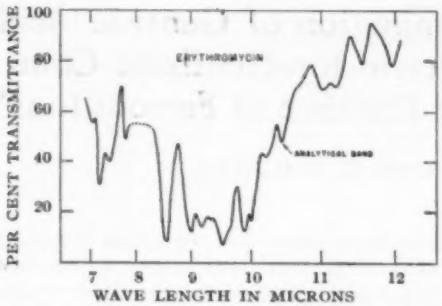

Fig. 1.—Infrared absorption spectrum of erythromycin in chloroform. (Dotted line indicates region where chloroform is total absorber.)

A Perkin-Elmer model 12-C infrared spectrometer equipped with sodium chloride optics and a three-speed slit drive was used in this study.

Procedure.—Transfer exactly 120 mg. of sample to a suitable flask, add 4.0 cc. of chloroform, stopper tightly, and swirl until solution is complete. Transfer the sample to a 0.5 mm. sealed fixed thickness cell and place in position for absorption measurement. Adjust amplifier gain setting so that a 2 microvolt test signal gives full scale deflection on the recorder. Using a slit width of .42 mm., and a wave-length drive speed of one turn/two minutes, scan the spectrum from 10.3–10.7 μ. Determine the absorption at 10.46 μ using the base line method (1). Refer the value to the working curve, and determine per cent purity of the sample. A qualitative spectrum may be run from 7–12 μ for the purpose of establishing identity.

DISCUSSION

Maximum solubility of erythromycin in chloroform is about 60 mg./cc. For practical purposes solutions at a concentration of 30 mg./cc. can conveniently be prepared with a minimum of loss due to evaporation. The base line method was selected in preference to the single spot determination since the former is relatively insensitive to moderate amounts of extraneous substances.

In the development of the method, four absorption bands common to erythromycin were investigated. The maxima of these bands were located at 7.29, 9.02, 9.88, and 10.46 μ. A study of these bands under the general conditions described in the procedure revealed that of the four bands, the one at 10.46 μ adhered most closely to straight line relationship between concentration and absorbance, and agreed most consistently with the microbiological assay.

TABLE I.—RESULTS OF ANALYSES OF EXPERIMENTAL AND PRODUCTION LOTS OF ERYTHROMYCIN

Sample No.	Assay (Mcg./Mg.) Infrared	Assay (Mcg./Mg.) Microbiological
1	950	942
	945	
2	884	810
	882	
3	920	933
	910	
4	970	906
	950	
5	930	1008
	930	
6	920	968
	910	
7	860	870
8	950	900
9	940	934
10	879	860
11	895	820
12	910	918
13	715	712
14	450	522

Comparison of analyses on various lots of erythromycin by both infrared and microbiological assays is shown in Table I. The microbiological assays[1] were carried out by a paper disk plate procedure (2) using *B. subtilis* as the test organism.

SUMMARY AND CONCLUSIONS

1. An infrared method has been developed for the determination of erythromycin.
2. The method is simple and rapid to perform; an analysis can be completed within one-half hour.
3. Reproducibility is in the range of ±1 per cent.
4. The method is specific in that erythromycin may be identified by its characteristic infrared absorption.

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[1] For these assays the author is indebted to the Abbott Microbiological Laboratories.

A Comparison of the Determination of Gentisic Acid in Blood by Means of the Ferric Ion-Gentisate Complex With and Without the Presence of Ferrous Ions*

By MARY CASS† and JOHN G. WAGNER‡

Two methods for the determination of gentisic acid in blood have been compared and the data treated statistically. Almost identical results were obtained by the two methods. The method involving the use of a reagent containing ferric ions alone is shown to have definite advantages over the method involving the use of a reagent containing both ferrous and ferric ions.

WHEN A SOLUTION of gentisic acid is treated with a solution containing ferric ions a transient blue color is produced; this solution has a broad absorption maximum at 595 mμ; the blue color rapidly changes to yellow, and this solution has an absorption maximum at 410 to 420 mμ. Camelin, *et al.*, (1) reported the analysis of gentisic acid in serum and urine by a colorimetric method with ferric chloride after extraction with ether. Gerald and Kagan (2) reported that "ferric chloride alone is difficult to use for quantitative measurement of gentisic acid, principally because of rapid fading of color." These authors found that the initial blue color could be stabilized by use of a reagent containing ferric chloride and large amounts of ferrous chloride in 0.01 *N* HCl. Their assay for the determination of gentisic acid in Folin-Wu concentrates of serum depended on the use of the ferrous-ferric chloride reagent and on readings in a spectrophotometer at 595 mμ within one minute after addition of the iron reagent. Wagner (3) showed that gentisic acid in aqueous solution could be determined by use of a reagent containing 1 per cent w/v $Fe(NO_3)_3 \cdot 9H_2O$ in exactly 1 per cent HNO_3, provided the measurements were made after the initial blue color had faded to yellow and at the wave length of maximum absorption of the yellow solution, 410 to 420 mμ, or by the use of a photelometer with a blue filter with maximum transmittance in this region.

Recent experiments in this laboratory indicate that almost identical results are obtained by the method of Gerald and Kagan (2) and by the method of Wagner (3) when applied to the determination of gentisic acid in tungstate filtrates of whole bovine blood. The statement of Gerald and Kagan that "inhibition of the oxidation of gentisic acid by ferric chloride was found to be essential" appears therefore to be erroneous.

There are definite advantages in the use of the ferric nitrate reagent. In the concentration range 6.25 to 87.5 μg. of gentisic acid per ml. of final colored solution, corresponding to 0.125 to 1.75 mg. of gentisic acid per ml. of blood, the blue color which developed with the ferrous-ferric chloride reagent is unstable; readings must be made within one minute at a wave length of 595 mμ. In the same concentration range, using the ferric nitrate reagent, the blue color fades rapidly but the intensity of the resulting yellow color is more stable and permits readings from one to eight minutes after the addition of the iron reagent. The ferrous-ferric chloride reagent is unstable and must be standardized within twenty-four hours of the time of use, whereas the ferric nitrate reagent is stable for long periods of time if refrigerated. Hence much time is saved by the use of the ferric nitrate reagent and repeated calibration is unnecessary. No turbidity developed when using the ferric nitrate reagent, but Gerald and Kagan stated this happened occasionally with their reagent. It appears to us that the development of turbidity may be due to the incomplete removal of proteins from the blood. This can be prevented by thorough agitation, both after addition of the sulfuric acid and after addition of the sodium tungstate, and by removal of the precipitated proteins by filtration rather than by centrifugation. Gerald and Kagan stated that their method cannot be applied to urine since a precipitate is formed immediately after addition of the ferrous-ferric chloride reagent. Samples of urine which we treated with ferric nitrate reagent gave no precipitate but no quantitative studies were made with urine.

EXPERIMENTAL

Reagents.—(*a*) Ferric nitrate reagent of Wagner (3). (*b*) Ferrous-ferric chloride reagent of Gerald and Kagan (2). (*c*) Sodium tungstate solution: 9.75 Gm. of sodium tungstate per 100 ml. of water.

* Received August 3, 1953, from College of Pharmacy, The Ohio State University, Columbus, Ohio.

† Graduate student, College of Pharmacy, The Ohio State University.

‡ Assistant Professor, College of Pharmacy, The Ohio State University. Present address: The Upjohn Company, Kalamazoo, Mich.

TABLE I

Concentration of Gentisic Acid, Mg./Ml. of Blood	—Beckman Spectrophotometer, Model DU— No. 1 Ferric Nitrate Reagent % T (at 410 Mμ)	No. 2 Ferrous-Ferric Chloride Reagent % T (at 595 Mμ)	—Cenco-Sheard-Sanford Photelometer— No. 3 Ferric Nitrate Reagent % T (Filter 410)	No. 4 Ferrous-Ferric Chloride Reagent % T (Filter 610)
0.000	100	100	100	100
0.125	...	...	81	...
0.250	80	81	78	77
0.375	...	...	73	...
0.500	70	66	63	64
0.625	...	...	56	...
0.750	50	54	52	49
0.875	...	...	46	...
1.00	46	48	41	35
1.25	33	34	35	30
1.50	28	28	...	...
1.75	23	22	...	...

TABLE II

No.	Equation of "Least-Squares" Regression Line	Prediction of $\hat{C}$ from Log % T for Unknown	Estimated Standard Error of $\hat{C}$[a]
1	Log % T = 2.003–0.3697 C	$\hat{C}$ = 5.419–2.705 log % T	0.067
2	Log % T = 2.009–0.3727 C	$\hat{C}$ = 5.390–2.683 log % T	0.061
3	Log % T = 1.980–0.3579 C	$\hat{C}$ = 5.533–2.794 log % T	0.038
4	Log % T = 2.002–0.4293 C	$\hat{C}$ = 4.664–2.329 log % T	0.055

C = Concentration of gentisic acid expressed in milligrams per milliliter of blood.

[a] The estimated standard error of $\hat{C}$ is made by using an approximate formula and assuming one determination of log % T per unknown. It could be reduced by making more than one sample determination of % T.

(*d*) Sulfuric acid: $^1/_2$ *N*. (*e*) Gentisic acid solution: 250 mg. of purified gentisic acid was dissolved in sufficient distilled water to make 100 ml. of solution.

Procedures.—The tungstate filtrates of whole bovine blood were prepared according to the method of Gerald and Kagan (2), except that the proteins were removed by filtration instead of by centrifugation. Various pairs of calibration curves were obtained by adding increments of a known solution of gentisic acid to citrated bovine blood and using equal parts of the tungstic acid filtrate and the two reagents. The blank used in all cases was a mixture of equal parts of tungstic acid filtrate (containing no gentisic acid) and the reagent concerned.

Measurements were made with a Beckman Spectrophotometer, Model DU, at a wave length of 595 mμ when the ferrous-ferric chloride reagent was used and at a wave length of 410 mμ when the ferric nitrate reagent was used. Measurements were made with a Cenco-Sheard-Sanford photelometer, using a filter with a maximum transmittance at 610 mμ when the ferrous-ferric chloride reagent was used and a filter with a maximum transmittance at 410 mμ when the ferric nitrate reagent was used. Comparisons were always made by the two methods on the same tungstic acid filtrates.

Results.—The data are summarized in Table I. The log % T values were plotted on the ordinates and the concentration values on the abscissa. The data were treated statistically by the method of covariance (4) and F tests were applied. The equations of the least-squares regression lines, equations to give an estimated value of concentration, $\hat{C}$, for an unknown when log % T is determined, and the estimated standard errors of $\hat{C}$ are summarized in Table II.

DISCUSSION

The statistical treatment indicated that for the Beckman spectrophotometer procedures, numbers 1 and 2, the regression lines are almost identical and do not differ significantly. The regression lines, numbers 3 and 4, obtained by the two-assay procedures with the Cenco-Sheard-Sanford photelometer have slopes which do differ significantly. The estimated standard errors of $\hat{C}$ by either method are almost the same and compare favorably with that reported by Gerald and Kagan (2).

Because of the outstanding advantages of using the ferric nitrate reagent over that of the ferrous-ferric chloride reagent it is recommended that the former is the best reagent and that the presence of ferrous ions is unnecessary in order to determine gentisic acid by means of the ferric ion-gentisate colored complex.

SUMMARY

1. A comparison between the method of Gerald and Kagan (2) and the method of Wagner (3) for the determination of gentisic acid was made.

2. The data were treated statistically. Almost identical results were obtained by the two methods over the concentration range 0.125 to 1.75 mg. per ml. of blood.

3. The method of Wagner offers outstanding advantages over the method of Gerald and Kagan. These are: (*a*) the reagent used in the

method of Wagner is stable for long periods of time if refrigerated, whereas the reagent used in the method of Gerald and Kagan is unstable and must be standardized within twenty-four hours of the time of using; (*b*) the color stability is greater by the method of Wagner and permits readings from one to eight minutes after addition of the reagent, whereas in the method of Gerald and Kagan readings must be made within one minute.

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The *in Vitro* Antifungal Activity of a Series of Acridine Salts*

By STANLEY M. VISCIA and DONALD C. BRODIE†

A series of salts of 9-aminoacridine with various anions, including fatty acids, have been prepared and tested for antifungal activity against *Trichophyton mentagrophytes*. In the salts prepared with fatty acids, the antifungal action paralleled the length of the carbon chain of the anion, with maximal activity occurring with the acridine caprate, undecylate, and undecylenate.

THE STUDIES of Albert, Rubbo, *et al.* (1), on members of the monoaminoacridine series of antibacterial agents provide considerable information regarding activity, toxicity, and mode of action of some 65 individual compounds. In these studies 9-aminoacridine (9AA) was found to be one of the most promising because of its potent antibacterial activity against both Gram-positive and Gram-negative organisms. The biological activity of the acridines is due to the cation and providing that the extent of its ionization "is not allowed to fall below about 50 per cent at *p*H 7.3, the chemical nature of the substituents in the acridine nucleus makes little difference" (2). Furthermore, a positive correlation between the antibacterial action and the degree of ionization has been shown in both the monoamino and diamino series of isomers (1).

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† The authors wish to thank Professor Robertson Pratt and Miss Grace Gardner for testing the compounds, Mr. Howard Fifer for preparing the 9-aminoacridinesorbate, and Professor Warren D. Kumler for reading the manuscript. The El Dorado Oil Works of Oakland, Calif., supplied capric, caprylic, lauric, and myristic acids of relatively high purity, and the Baker Castor Oil Company of Los Angeles supplied the undecylic acid.

9-Aminoacridine is ordinarily used as the hydrochloride (9AAHCl); in this form it is approximately 70 per cent ionized at 20° (50 per cent at 37.5°), *p*H 7.2, which assures a potent antibacterial action. Because the compound produces considerable necrotic damage (3, 4) when applied to abraded tissues or when it is injected, its use has been confined almost entirely to local application.

Daniels, McOmey, and Brodie (5) observed that 9-aminoacridinelaurylsulfate (9AALS) possesses as great or greater antibacterial activity than the hydrochloride during the course of formulation studies in these laboratories, and the compound has been used since as an effective prophylactic agent in the control of human mastitis at the University of California Hospital. These observations prompted the preparation of a series of salts of 9AA with fatty acids. *In vitro* testing of these and a number of miscellaneous salts revealed not only antibacterial, but also a surprising degree of antifungal activity. This paper reports the results of the antifungal studies. The following salts of 9AA were prepared and tested: acetate, propionate, butyrate, caproate, capry-

late, caprate, undecylate, undecylenate, laurate, laurylsulfate, myristate, palmitate, stearate, oleate, benzoate, salicylate, and sorbate.

EXPERIMENTAL

Salts were prepared by dissolving the free acid in a suitable solvent and adding to it slowly an equivalent amount of 9AA dissolved in the same solvent. Since 9AALS had been observed previously to be heat labile, heat was restricted to 50° in both preparation and recrystallization procedures. Absolute alcohol was used as the solvent in the preparation of salts of acids with chain lengths of C-12 or greater. In all cases except the salt of C-12, the salt precipitated in crystalline form immediately upon mixing the reactants. The laurate was recovered by reducing the amount of solvent *in vacuo* at 40°. For salts of acids less than C-12, acetone was used as solvent and again crystalline precipitates were obtained when the two solutions were mixed. The benzoate and salicylate were not recovered in the crystalline state. Recrystallization of all salts was obtained through the use of alcohol, acetone, dioxane, or petroleum ether. Yields of crude product varied from 82–91% of the theoretical. Sharp melting points could not be obtained and decomposition usually occurred between 190 and 196°.

Antifungal tests were made by incorporating a previously prepared dilution of the drug in Sabouraud's agar medium. After transferring the medium to plates, it was streaked with a seven-day culture of *Trichophyton mentagrophytes*. Antifungal activity was determined by comparing the density of growth in experimental and control plates after a seven-day incubation period at 25°.

Since the salts had limited solubility in water, thereby necessitating the use of alcoholic solutions for testing purposes, 1–300 stock solutions of each salt in 95% alcohol were prepared. From these, appropriate dilutions with alcohol were made prior to the time that the compounds were added to the agar. Hence, the amount of alcohol in all experiments remained constant. Control plates contained a similar amount of alcohol. The minimal concentration of all drugs in the medium was 1–10,000 excepting the benzoate and salicylate which were 1–16,600. All concentrations are expressed in terms of the cation for the salts of 9AA and in terms of the anion for the fatty acids or salts thereof.

For results see Fig. 1.

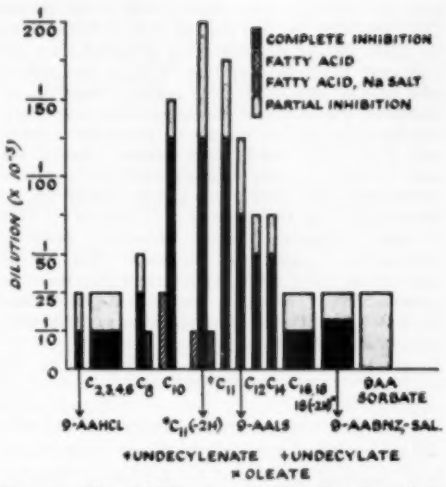

Fig. 1.—The inhibitory activity of salts of 9-aminoacridine against *Trichophyton mentagrophytes*.

DISCUSSION

The inhibitory action of fatty acids and salts of fatty acids against fungi was first observed by Clark (6) in 1899, and since that time a rather extensive literature (7) has developed, testifying to the antibacterial as well as to the antifungal behavior of these compounds. As has been demonstrated in homologous series of other compounds, the inhibitory action of members of the fatty acid series parallels the length of the carbon chain with maximal activity often occurring with chain lengths of 10, 11, and 12 carbon atoms. The point of cut-off in a given homologous series depends upon the resistance of the organism, and it has been observed that the maximum occurs earlier in a series the greater the resistance of the organism (8). Factors which have been correlated with biological activity in homologous series are water solubility, the partition between immiscible phases, vapor pressure and capillary activity. Perhaps the most scientific evaluation of the factors contributing to this behavior of homologous series is that set forth by Ferguson (8). Ferguson accounts for this phenomenon by identifying the cutoff or maximum, as the point of intersection of the logarithm of equitoxic concentrations of the members of the series with the logarithm of their solubilities ("saturation line") when they are plotted against the logarithm of their solubility. An increase in biological effect with an increase in methylene groups in an homologous series does not signify a greater inherent toxicity with each succeeding member. It does, however, reflect a physical phenomenon due to an alteration in partition coefficient between the surrounding medium and the biological medium. When a point is reached in a given series beyond which a decrease in biological activity is obtained, the solubility of the individual members is less than the predictable lethal concentration.

The data reported in Fig. 1 are therefore in keeping with the known pattern for biological activity of members of homologous series. In the present acridine series it is obvious that the anion contributes a significant biological effect since the degree of inhibition depends upon the length of the carbon chain of the fatty acid. Whereas 9AAHCl has been the most commonly used antibacterial agent of the acridine series, its degree of antifungal action is relatively low when compared to that of the fatty acid salts on the basis of equimolar concentrations of cation (9AA), with maximal activity occurring from the C-10 to C-12LS.

In order to eliminate the likelihood that the antifungal activity was due to the anion, the fatty acid

and its corresponding sodium salt were tested. This was done in the case of C-8, C-10, and C-11(-2H). Slight inhibition was obtained from the C-10 and C-11(-2H) acids and from the sodium salts of C-8 and C-11(-2H) acids. However, no inhibition was obtained at the minimal concentration for either sodium caprate (C-10) or for caprylic acid (C-8). The above data indicate that in the cases of the acridine caprate and undecylenate particularly, the antifungal action cannot be attributed to either the anion or the cation or the sum of their respective effects. Therefore, there is evidence to support the statement that in the cases of the most active members of this series, a potentiation of effect is obtained beyond that attributed by either the fatty acid or acridine portion of the molecule.

SUMMARY

1. A series of salts of 9-aminoacridine with various anions, including the fatty acids, have been prepared and tested for antifungal activity against *Trichophyton mentagrophytes*.

2. In the salts prepared with fatty acids, the antifungal action paralleled the length of the carbon chain of the anion, with maximal activity occuring with the acridine caprate, undecylate, and undecylenate.

3. When compared with the antifungal activity of equivalent amounts of the anion and of the cation, it appears that the action of the salt is a potentiation of the component effects, at least with the most active compounds.

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Aralkyl Dithiobiuret Analogs of Tibione*

By SAMUEL B. BRITTON and W. L. NOBLES†

A new type of aralkyl ketone derivative has been obtained by condensing the desired ketone with dithiobiuret to achieve the monosubstituted product. Interest in this type of compound centers around the structural relationship existing between nuclear substituted acetophenone thiosemicarbazones, which have *in vitro* antitubercular activity, and the corresponding dithiobiurets.

ONE OF THE MOST active groups of synthetic tuberculostats known today is the thiosemicarbazones (1). The announcement by Domagk (2) of the antitubercular activity of the *para*-substituted benzal thiosemicarbazones(I), of which Tibione (*p*-acetylaminobenzaldehyde thiosemicarbazone) was most promising, has evoked much interest in the synthesis of related structures. Scores of variations have been made in the structure of Tibione in an effort to increase activity and decrease toxicity. Most of the changes have involved the aryl group, since earlier workers in the field had pointed out that substitution of the thiosemicarbazone moiety by the semicarbazone, anil, oxime, hydrazone, azine, and similar groups abolished the activity (3). Despite this conclusion it was decided to prepare some dithiobiuret analogs of Tibione from aralkyl ketones, since much less attention has been given to the side-chain modification of this class of compounds than to changes in the aryl nucleus.

A recent report (4) also indicates that the thiosemicarbazone of 4-acetylpyridine possesses marked *in vitro* antitubercular activity. Furthermore, most of the side-chains previously used have been neutral or basic, but the dithiobiuret group (II) would confer acidic properties on the molecule. Furthermore, interest in this type of compound was increased as a result of the *in vitro* antitubercular activity reported for the aldehyde dithiobiurets reported by Foye and Hefferren (11).

* Received August 21, 1953, from the School of Pharmacy, University of Mississippi, University, Mississippi.
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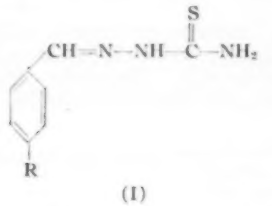
† Assistant Professor of Pharmacy and Pharmaceutical Chemistry, University of Mississippi, University.

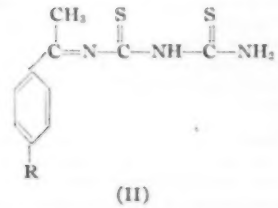
TABLE I.—DITHIOBIURET DERIVATIVES OF KETONES

No.	Ketone Component[a]	Yield, %	M. P.[b]	Formula	Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	*p*-Chloropropiophenone[d]	17	218	$C_{11}H_{12}ClN_3S_2$	46.23	45.86	4.23	4.01
2	Acetophenone[d]	32	210	$C_{10}H_{11}N_3S_2$	50.60	50.01	4.67	4.69
3	*p*-Chloroacetophenone[c]	21	240	$C_{10}H_{10}ClN_3S_2$	44.18	44.79	3.71	3.28
4	*p*-Nitroacetophenone[d]	26	235	$C_{10}H_{10}N_4O_2S_2$	42.54	42.25	3.57	3.22
5	*p*-Ethoxyacetophenone[e]	19	234	$C_{12}H_{15}N_3OS_2$	51.41	50.92	5.39	4.96
6	2-Acetylthiophene[e]	22	219	$C_8H_9N_3S_3$	39.48	39.86	3.73	3.95

[a] Compound 2 is off-white in color; 1, 3, 4, 5 are various shades of yellow; and 6 is orange.
[b] Melting points are uncorrected.
[c] Intermediate ketones were obtained from the Eastman Kodak Company.
[d] Ketone was obtained through the courtesy of the Monsanto Chemical Company.
[e] Ketone was obtained from the Arapahoe Chemicals Company.



(I)



(II)

Although derivatives of dithiobiuret have been known for more than eighty years (5–7), the parent substance was first prepared by Sperry (8) in 1945. He (9) condensed it with formaldehyde to yield 1,5-bis(hydroxymethyl)-dithiobiuret. Likewise, cyclohexanone has been condensed with it to yield the dialkylidene derivative (10). Shortly after this work was begun, Foye and Hefferren (11) reported the preparation of several monosubstituted dithiobiuret derivatives of various benzaldehydes. Thus far, no other products of this type have been reported.

The dithiobiurets described in Table I are to be tested for their tuberculostatic activity.

EXPERIMENTAL[1]

Ketone Condensations with Dithiobiuret.—The following procedure is similar to the general method of Foye and Hefferren (11), but differs in certain details. Thirteen and one-half grams (0.1 mole) of dithiobiuret[2] was dissolved in 125–200 ml. of glacial acetic acid heated to reflux temperature. After a clear solution had been obtained, 0.2 mole of ketone was added. The resulting solution was refluxed for twelve to eighteen hours, cooled to room temperature, and then chilled until crystals formed. In the case of *p*-nitroacetophenone a solid formed in the flask during the refluxing. The solids thus obtained were removed by filtration, washed with dilute alcohol and water, recrystallized from glacial acetic acid, and dried in a vacuum desiccator at room temperature. The compounds thus obtained were of analytical purity.

The products were insoluble in water and only slightly soluble in the common organic solvents. All the products were soluble in dilute sodium hydroxide solution.

[1] Microanalyses by Weiler and Strauss, Oxford, England.

SUMMARY

A new type of ketone derivative has been prepared by condensing dithiobiuret with several aralkyl ketones. The substituted dithiobiurets were prepared using *p*-chloropropiophenone, acetophenone, *p*-chloroacetophenone, *p*-nitroacetophenone, *p*-ethoxyacetophenone, and 2-acetylthiophene.

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[2] The authors wish to thank American Cyanamid Company for the generous supply of dithiobiuret for use in this work.

A Survey of Fungi from the Order *Melanconiales* for Antibiotic Activity*

By FRANKLIN R. COLE and RAYMOND W. VANDERWYK†

Twenty-six genera including 55 different species of fungi from the order *Melanconiales* were tested for antibiotic activity. A comparison of the value of four broth substrates used in this survey is given. Twenty-three species were found to be active. More species were found to be active against Pseudomonas *aeruginosa* than any other test organism used. None of the molds was found to be active against *Bacillus subtilis* or *Escherichia coli*.

THE *Melanconiales* are a form-order of the form-class *Deuteromycetes*. The group consists of one family, the *Melanconiaceae*, which includes over 600 species in 77 (1) to 92 (2) genera. Many members of the order are plant parasites which cause serious plant diseases. The *Melanconiales* are an heterogenous order of molds, the members of which are considered to be related because their conidia are borne in acervuli. This characteristic, used in classifying the group, is a purely artificial one. No phylogenetic relationship has been shown to exist among members of this order.

According to Brian (3) there is no evidence in the literature that members of the *Melanconiales* have ever been screened for antibiotic activity. A search of the literature showed this statement to be correct.

Each of 55 species, distributed in 26 genera of the *Melanconiales*, was tested against eight known strains of pathogenic bacteria by the agar cup-plate method. In all, 87 cultures were tested. These included some which had only been identified as to genus, as well as some strain variants and some duplicate species. Of the 55 different species tested, 23 showed antibiotic activity against one or more of the test bacteria. However, none was found to be active against *Escherichia coli* or *Bacillus subtilis*.

MATERIALS

Fungus Cultures.—Cultures of Melanconiaceous fungi were obtained from various State agricultural experiment stations in the United States, from colleges, and universities associated with them, from the Nagao Institute in Tokyo, Japan, and the Centraalbureau voor Schimmel-cultures in Baarn, Holland. The scientific names used in designating these cultures are the ones used by the donors of the cultures.[1]

Bacterial Cultures.—All bacterial cultures were obtained from the National Type Culture Collection in Washington, D. C. The numbers following the name of each bacterium indicate the specific strain used.

Aerobacter aerogenes	8308
Bacillus subtilis	6051
Escherichia coli	26
Klebsiella pneumoniae	Type A 8045
Proteus vulgaris	8427
Pseudomonas aeruginosa	9027
Salmonella typhosa	F. D. A. Hopkins 6539
Micrococcus pyogenes var. *aureus*	F. D. A. 209

Broth Media.—For the determination of the best substrate for the growth of Melanconiaceous fungi experimental batches of four different broths were prepared and tested. These included Czapek-Dox broth, modified Mycophil[2] broth, prune decoction broth, and corn meal infusion broth.

Czapek-Dox Broth.—

Sucrose	30.0
Sodium nitrate	3.0
Dipotassium phosphate	1.0
Magnesium sulfate	0.5
Potassium chloride	0.5
Ferrous sulfate	0.01
Distilled water	1,000.0
Hydrochloric acid	A sufficient quantity
Final pH	5.5

The salts were dissolved in water and enough hydrochloric acid was added to adjust the *p*H to 5.5. The broth was sterilized at 15 pounds' pressure for fifteen minutes.

Modified Mycophil Broth.—

Bacto peptone	5.0
B. B. L. trypticase	5.0
Dextrose	40.0
Distilled water	1,000.0
Final pH	5.8 to 7.2

The dry materials were dissolved in water and brought up to volume. The broth was sterilized for fifteen minutes at 15 pounds' pressure.

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Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
Abstracted from a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by Franklin R. Cole, Fellow in Pharmacognosy, Massachusetts College of Pharmacy, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
† Associate Professor of Biology and Pharmacognosy, Massachusetts College of Pharmacy.

[1] A detailed list of all fungi used in these experiments is deposited in the Sheppard Library of the Massachusetts College of Pharmacy.
[2] Mycophil broth is commercially available from Baltimore Biological Laboratories, Baltimore, Md.

TABLE I.—COMPARISON OF BROTH SUBSTRATES

	Prune	Corn Meal	Modified Mycophil	Czapek-Dox
Mold Growth	Good	Fair	Good	Good
Inhibition of test bacteria	All	None	One (*E. coli*)	All but *S. typhosa*
Sterilization characteristics	Good	Good	Good	Good
Nitrogenous substances	Little	Little	Plentiful	Little
Convenience of preparation	Good	Poor	Excellent	Excellent

Prune Decoction Broth.—

Prunes	200.0
Distilled water	1,000.0
Final pH	4.0

The prunes were boiled for about an hour, strained and enough water added to measure one liter. The broth was sterilized at 15 pounds' pressure for fifteen minutes.

Corn Meal Infusion Broth.—

Yellow corn meal	100.0
Dextrose	2.0
Distilled water	1,000.0
Final pH	7.0

The corn meal was infused with boiling water for one hour. It was strained and the dextrose added. The broth was sterilized at 15 pounds' pressure for fifteen minutes.

EXPERIMENTAL

Of the several culture media described above, modified Mycophil broth was selected because it proved to have several advantages over the other media which were tried. For example, prune broth and Czapek-Dox broth both inhibit the growth of most of the test organisms, while the mold growth on corn meal infusion is not as luxuriant as that on modified Mycophil broth. Furthermore, the presence of amino acids in the formula of modified Mycophil broth may enhance the possibility of antibiotic production since many antibiotics contain nitrogen in the molecule.

Table I summarizes the advantages and disadvantages of the four broths used in this work.

Agar Cup Plate Method.—Approximately 1 ml. of a twenty-four hour broth culture of each of the test organisms used was added to 100-ml. lots of sterile melted nutrient agar which had been cooled to about 45°. After being mixed thoroughly, the seeded agar was poured into sterile Petri plates and was allowed to solidify. Four cups were cut in each agar plate with a sterile No. 6 cork borer, which produced cups of about 11 mm. diameter.

About 0.5 ml. of broth substrate from each growing fungus culture was placed in one of the cups by means of a sterile pipet. Plates so prepared were incubated at 37° for twenty-four hours after which the zones of inhibition, if present, were measured.

Cultivation of Fungi.—The fungus cultures were grown at 28° during the period of the test. Tests were made at the end of five- and twelve-day growing periods.

The fungi were grown in ordinary six-ounce prescription bottles containing about 50 ml. of broth. To obtain a greater surface area the bottles were incubated at an angle of about 30°. The containers were plugged with cotton and handled aseptically at all times.

Sterile broth substrate was used as the control in all determinations.

RESULTS

Zones of inhibition are reported in millimeter diameters. The measurements include the diameter of the agar cup. Zones were considered to be complete if absolutely no growth could be observed in the measured area. A partial zone of inhibition was considered to be one in which a few scattered colonies were found within the measured area. All zones reported in the following tables may be assumed to be complete unless otherwise noted. Both the partial and complete zones of inhibition have shown a definite ring of stimulated growth around their circumferences.

If a zone of inhibition, either complete or partial, could be observed next to the cup at any test age, the mold was considered to exhibit antibiotic activity.

TABLE II.—SPECIES ACTIVE AGAINST PSEUDOMONAS AERUGINOSA

Species	Age of Fungus: 5 days	Age of Fungus: 12 days
Amphichaeta musciocola	16[a]	0[b]
Asterosporium Hoffmanni	0	13[c]
Colletotrichum atramentarium	0	13
C. destructivum	13[c]	18[c]
C. lindemuthianum, β-strain	0	13[c]
C. lindemuthianum, (E-77-2)	0	21[c]
C. linicolum	0	20[c]
C. trifolii from alfalfa	0	25[c]
C. trifolii isolate $1.	0	21[c]
C. trifolii isolate $2.	0	22[c]
C. trifolii isolate $3.	0	17
Coryneum Kunzii	0	19
Cryptomela typhae	0	18[c]
Cryptostictus caudatus	0	20[c]
Diploceras hypericinum	19	0
Entomosporium maculatum	21	16
Gloeosporium concentricum	16	0
Glomerella cingulata	21	0
Libertella acerina	0	18
Marssonina graminicola	0	16
Pestalotia funerea	16[c]	0
Pseudodiscosia avenae	0	20[c]
Polyspora lina	0	13[c]
Steganosporium pyriforme	0	16[c]
Sterile control	0	0

[a] Zones of inhibition in mm. diameter.
[b] No inhibition.
[c] Partial zone of inhibition.

TABLE III.—SPECIES ACTIVE AGAINST TEST ORGANISMS OTHER THAN PSEUDOMONAS AERUGINOSA

Species of Fungus	Age of Fungus in Days	BACTERIAL TEST ORGANISMS				
		Klebsiella pneumoniae	*Proteus vulgaris*	*Salmonella typhosa*	*Micrococcus pyogenes var. aureus*	*Aerobacter aerogenes*
Cryptostictus caudata	5	0[b]	30[a]	20	17	0
	12	0	0	0	0	0
Marssonina melonis	5	19	21	0	17	17
	12	0	0	0	0	0
Neofabrea perennans	5	0	35	0	0	0
	12	0	0	0	0	0
Sterile control	5	0	0	0	0	0
	12	0	0	0	0	0

[a] Zones of inhibition in mm. diameter.
[b] No inhibition.

Those species of fungi which showed antibiotic activity are arranged in Tables II, III, and IV. Because there were a great many more fungi which showed activity against *Pseudomonas aeruginosa* than any other test bacteria, they are listed separately in Table II.

Those few species that were found to be active against other test bacteria are listed in Table III. A summary of activity against all test bacteria is found in Table IV.

TABLE IV.—SUMMARY OF ANTIBIOTIC ACTIVITY AGAINST BACTERIA USED

Gram-Negative Bacteria	
Aerobacter aerogenes	1 species
Escherichia coli	None active
Klebsiella pneumoniae	1 species
Proteus vulgaris	3 genera
	3 species
Pseudomonas aeruginosa	16 genera
	20 species
Salmonella typhosa	1 species
Gram-Positive Bacteria	
Bacillus subtilis	None active
Micrococcus pyogenes var. *aureus*	2 genera
	2 species

CONCLUSIONS

It was shown that some species of the order *Melanconiales* are antibiotically active, particularly against the pathogen *Pseudomonas aeruginosa*.

The molds *Cryptostictus caudata* and *Marssonina melonis* produce a deep red pigment after about seven days' growth. Although these species show activity against more of the bacteria used than do any other molds, they do not inhibit *P. aeruginosa*. Those molds which inhibit *P. aeruginosa* do not act against any of the other bacteria used. There may be some connection between the presence of a red pigment and the lack of activity against *P. aeruginosa*.

It is felt that although there are certainly antibiotic substances present in the active molds, the amounts produced are probably too minute to be significant from an economic point of view. The chief value of this survey seems to be in the ability of some members of the group to inhibit *P. aeruginosa*, an organism that has been shown to be resistant to many of the commercially available antibiotics.

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The Preparation of Some Amides of Dichloroacetaldehyde*

By WILLIAM D. EASTERLY† and JOSEPH P. LaROCCA‡

The amides of dichloroacetaldehyde were synthesized and their physical and chemical constants were determined. None of the compounds showed any appreciable hypnotic or analgetic activity. Two of the compounds, the isovaleramide and 2-ethylbutyramide derivatives showed some anticonvulsant activity.

VARIOUS substituted amides have been reported in the literature. Jacobsen (1) prepared the chloral derivatives of acetamide, benzamide, and urea. Other chloral derivatives reported are those of formamide (2) propionamide (3), valeramide (3), caproamide (3), and caprylamide (3). Schiff and Tassinari (4) synthesized the α, α, β-trichlorobutyraldehyde derivatives of acetamide, ammonia, and benzamide.

LaRocca, *et al.* (5), synthesized 24 chloral and α, α, β-trichlorobutyraldehyde amides, 18 of these compounds being new. These compounds were made by condensing on a steam bath equimolar portions of chloral hydrate (or α, α, β-trichlorobutyraldehyde) and the appropriate aliphatic amide, the condensation requiring one to eight hours. The condensed solids were washed with water to remove any unreacted starting materials and recrystallized repeatedly from dilute alcohol until melting points were constant. The products were then dried over phosphoric anhydride *in vacuo*. All of these compounds were white crystalline solids, insoluble in water, but soluble in alcohol and acetone. Purification was tedious due to the difficulty in removing traces of unreacted amide from the final product. In general, the α, α, β-trichlorobutyraldehyde amides melted somewhat higher than the chloral derivatives.

Work with these various amide derivatives has shown their potentialities as sedative-hypnotics. Byrum and LaRocca (6), investigated the chloral and α, α, β-trichlorobutyraldehyde amides for sedative-hypnotic action, determining the HD_{50} and LD_{50} for 19 different compounds. They found chloral butyramide, chloral isovaleramide, chloral 2-ethylcaproamide, and chloral 2-ethylbutyramide to be the most active. These compounds compared favorably with chloral hydrate as to milligram potency, therapeutic ratio, and duration of action.

Substituted amides have also shown potentialities as fungicides. Leonard and Blackford (7) made a study of the α-bromoacetamides and reported remarkable levels of fungus inhibition. LaRocca, Leonard, and Weaver (5) tested some chloral and α, α, β-trichlorobutyraldehyde amides for fungicidal activity, but their preliminary assays indicated these compounds have little activity as fungicides.

In view of the favorable results obtained in the studies of amide derivatives, it was decided that the synthesis of some dichloroacetaldehyde amides should be attempted in order that further studies on substituted amides could be made.

EXPERIMENTAL

Materials.—Many of the intermediates used in this investigation were purchased through ordinary commercial sources. The aliphatic acids used in preparing the acid chlorides were purchased from Eastman Kodak Company. Westvaco Chemical Company supplied the dichloroacetaldehyde.

Synthesis of Intermediates.—The acid chlorides used in the preparation of the various amides were made by reacting the corresponding fatty acid with PCl_3, using a ratio of three moles of fatty acid to one mole of PCl_3. The reacting mixture for each acid chloride preparation was placed in a beaker and heated on a water bath for approximately one minute, or until cloudiness occurred. Upon placing the mixture in an ice bath, phosphorus acid in the form of a viscous liquid settled to the bottom of the beaker. The acid chloride was then removed by decanting.

Various of the amides used were prepared by adding the appropriate acid chloride, drop by drop, from a separatory funnel into a concentrated ammonium hydroxide solution cooled by a dry ice and acetone bath. The precipitate of amide was filtered off with suction and purified by dissolving in hot benzene and filtering again while hot. The hot filtrate contained the amide which crystallized out immediately upon cooling. This method of preparation was not practical for some of the amides due to the violent reaction of certain of the acid chlorides with ammonium hydroxide solution and the difficulty in keeping the reacting temperature low enough for sizable yields to be obtained. In these instances the ammonium salt method of preparation was used, or else the amide was purchased through ordinary commercial sources.

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† Fellow of the American Foundation for Pharmaceutical Education, 1951 to 1952.

‡ The authors wish to acknowledge the generous financial assistance of the American Foundation for Pharmaceutical Education and Eli Lilly and Company.

TABLE I.—DICHLOROACETALDEHYDE AMIDES[a]

$RCONHCH(OH)CHCl_2$

Amide	Yield, %	M. P., °C.[b]	Nitrogen, Calcd.	Nitrogen, Found[c]	Chlorine, Calcd.	Chlorine, Found[c]
Acetamide	18	113	8.14	7.98	41.3	39.8
Propionamide	25	116	7.53	7.33	38.1	37.9
Butyramide	17	55	7.00	6.85	35.5	34.1
Isobutyramide	11	93	7.00	6.99	35.5	33.7
Valeramide	10	72	6.54	6.14	33.1	32.7
Isovaleramide	13	94	6.54	6.47	33.1	32.6
Caproamide	21	72	6.14	5.88	31.1	30.9
2-Ethylbutyramide	53	117	6.14	6.05	31.1	31.0
Benzamide	47	115	5.98	5.85	29.9	29.7
Phenylacetamide	32	92	5.64	5.63	28.6	28.3

[a] The *Chemical Abstracts* name for these compounds, N-(2,-2-dichloro-1-hydroxyethyl) amides was not used in this paper because it is unwieldy.
[b] All melting points on calibrated Fisher-Johns apparatus.
[c] Average of two determinations.

Depolymerization of Dichloroacetaldehyde.—Due to the tendency of dichloroacetaldehyde to polymerize and solidify, it was necessary to depolymerize much of it immediately before use. A modification of Fieser's method for depolymerizing paraldehyde (8) was employed.

Thirty grams of the polymerized dichloroacetaldehyde (solid) was placed in a 500-cc. round-bottomed flask fitted with a Claisen head, to which was attached a ground glass adapter and cooled condenser. Cotton was fitted into the space between the adapter and the receiver, a 250-cc. Erlenmeyer flask. A mixture of 1 cc. each of sulfuric acid and water was poured into the flask with the aldehyde, then heat applied. The dichloroacetaldehyde distilled at 85–88°. To avoid charring of the mixture, distillation was discontinued after about two-thirds of the material had been converted into the dichloroacetaldehyde monomer.

Synthesis of Dichloroacetaldehyde Amides.—One-tenth mole of the appropriate amide was allowed to react with 0.16 mole of dichloroacetaldehyde at room temperature (approximately 27°) until solidification occurred. The time required for this condensation varied from one to seven days. The paraffin-like solid was dissolved in boiling chloroform and this hot solution then filtered using a Büchner funnel with a high-grade filter paper. The filtrate was placed under a stream of compressed air until crystallization began. After twenty-four hours in the refrigerator the product was filtered, using a fritted glass crucible and suction. Recrystallization was repeated until the melting point was constant. The product was dried over calcium chloride *in vacuo*. The condensation of phenylacetamide with dichloroacetaldehyde was carried out in a refrigerator.

Analytical Methods.—Each compound was first tested qualitatively for nitrogen and chlorine by application of the sodium fusion process. Then quantitative determinations were made using the peroxide bomb fusion method for chlorine and a modification of the Kjeldahl method for nitrogen. The analytical data for ten new compounds are given in Table I.

Pharmacological Data.—These compounds were screened by Eli Lilly and Company for possible anticonvulsant, hypnotic, and analgetic activity.

Only two compounds showed any anticonvulsant activity. Dichloroacetaldehyde isovaleramide in oral doses of 400 mg./Kg. in rats gave 80% protection as determined by the Metrazol test method and 40% protection as determined by the electroshock method. The 2-ethylbutyramide derivative at the same dosage level gave 60% protection by the Metrazol test and zero protection by the electroshock method.

None of the compounds showed significant hypnotic or analgetic activity.

SUMMARY

The synthesis and physical properties of ten dichloroacetaldehyde amides have been described. All ten are new compounds.

The pharmacological screening of these compounds has shown the absence of significant hypnotic or analgetic activity. Two of the compounds, the isovaleramide and 2-ethylbutyramide derivatives show some anticonvulsant activity.

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The Isolation of a Material Capable of Producing Experimental Lathyrism*

By HAROLD P. DUPUY† and JORDAN G. LEE

A procedure for the isolation from Singletary pea (*Lathyrus pusillus*) seed of a crystalline, nitrogen-containing organic compound is given. This material produces in albino rats the syndrome associated with the ingestion of large quantities of the seed. On the basis of certain differences in properties it is suggested that more than one toxic material will eventually be isolated from the members of the genus.

THE INGESTION of seeds of the *Lathyrus* genus has long been recognized as the cause of the syndrome known as lathyrism. The various species of the genus differ in their toxicity and there is also a difference in response of the various test animals studied. Lewis and his group have provided the best comparative data (1, 2). There seems to be no doubt that the toxicity is associated with the presence within the seeds of a definite toxic material or materials. Claims have been made that the toxicity is due to the presence of phytin (3) or divicine (4). Evidence against these claims has been presented (1, 5).

The Singletary pea (*Lathyrus pusillus*) is widely grown in this area as a winter cover crop. When seeds of this species are fed to albino rats in large quantities the animals develop the progressive skeletal changes and muscular paralysis usually associated with experimental lathyrism in the rat (5). This investigation had as its object the isolation of the causative factor or factors present in the seed of the Singletary pea. An isolation procedure which yields such material in crystalline form is reported.

EXPERIMENTAL

Seven kilograms of Singletary peas were decorticated and comminuted. The mass was placed in a 5-gallon crock and covered with 12 L. of 50% ethanol. This mixture was stirred at intervals over a forty-eight hour period and then centrifuged in a basket centrifuge. The residual solid material was extracted and centrifuged as before. The combined extract was allowed to stand until a clear filtrate could readily be obtained. This was concentrated to a volume of 3 L. in a spray evaporator at 35–40°. The concentrate was highly active and contained 72 Gm. of dry matter per Kg. of peas extracted.

Decortication markedly decreased both dry matter and pigment content of the extract without loss of activity. The dilute ethanol was chosen because of ease of preparation. Temperatures here and throughout were kept below 50° because rapid loss of activity occurred at higher temperatures.

Two hundred milliliters of a saturated solution of neutral lead acetate was now added to the concentrated extract and the mixture was allowed to stand for six to twelve hours before filtration. The precipitate was carefully washed with distilled water. The filtrate and washings were pooled and deleaded with H_2S. The PbS was removed by filtration and washed with water. The pooled filtrate and washings were first aerated and then concentrated at 40–45° to a volume of 840 ml. This concentrate was highly active and contained 63 Gm. dry matter per Kg. of peas extracted.

The precipitation with lead ion was inserted to eliminate the formation of stubborn emulsions in the next step. The decrease in dry matter and pigment content which occurred were entirely secondary considerations.

A volume of concentrate equivalent to 2 Kg. of peas (240 ml.) was now added dropwise to 3 L. of 95% ethanol in a 6-L. Erlenmeyer flask and the mixture was shaken to insure dispersion. Three hundred milliliters of saturated K_2CO_3 solution were added and the mixture was thoroughly agitated and then allowed to stand until the phases separated. The ethanol phase was now removed, shaken with a second 300-ml. portion of saturated K_2CO_3 solution, and separated as before. The two K_2CO_3 fractions were now successively extracted with three 3-L. portions of 95% ethanol and discarded. The four ethanol solutions were carefully neutralized with 5 *N* H_2SO_4, filtered, pooled, and concentrated at 35° to a volume of 500 ml. The *p*H was again adjusted to 7. The concentrate was highly active and contained 30 Gm. of dry matter per Kg. of peas extracted.

This extraction drastically reduced dry matter without marked loss of activity. The final *p*H must be carefully controlled to avoid large losses in the next step.

The ethanol extract was now poured over 40 Gm. of pulped filter paper, dried in a current of warm air, and stored in a desiccator to remove traces of solvent. The dried material was divided into 6 portions which were extracted successively with 640 ml. of dry butanol by beating for fifteen minute periods in a Waring Blendor. The pulp was filtered off on a Büchner funnel with suction and re-extracted as before with a second portion of butanol. This butanol extract was inactive provided the *p*H had been adequately controlled in the previous step and contained 20 Gm. dry matter per Kg. of extracted peas.

* Received June 15, 1953, from the Department of Agricultural and Biochemistry, Louisiana State University, Baton Rouge.

† Abstracted from a thesis submitted to the Graduate Faculty of the Louisiana State University by H. P. Dupuy in partial fulfillment of the requirements for the degree of Master of Science.

The butanol removed inactive dry matter more efficiently than other solvents which were tried. The Waring Blendor proved to be more rapid and thorough than other feasible methods of extraction and left the pulp in such a fine condition that subsequent extraction with methanol was rapid and complete.

The butanol-extracted pulp was now placed in a Soxhlet type extractor and extracted *in vacuo* with absolute methanol for a period of twenty-four hours at a temperature of 25–30°. This extraction removed 8.5 Gm. of dry matter per Kg. peas. The extract, 1,320 ml., was now concentrated to a volume of 700 ml. at 30°. No precipitation occurred in the evaporator with this volume but when removed and allowed to stand at room temperature for forty-eight hours the solution spontaneously deposited the bulk of active material as a fine, yellowish crystalline solid. This precipitate weighed 7 Gm. when dry. The supernatant retained the remaining 10 Gm. of dry matter and was only slightly active.

Seven grams of this crude material was dissolved at room temperature in 150 ml. of 25% ethanol and decolorized with decolorizing carbon. The carbon was removed and twice washed with distilled water. The pooled filtrate and washings were concentrated at 40° to a volume of 39 ml. This was poured into 975 ml. of absolute ethanol and the solution was allowed to stand overnight at room temperature. The fine crystalline white solid which precipitated was dried in a desiccator. The dry product weighed 5 Gm. (2.5 Gm. per Kg. of extracted peas). The material could also be recrystallized with heat from distilled water or dilute ethanol solutions with greater losses of material and no apparent change in the crystalline product.

The recrystallized material, after drying in a vacuum oven at 40° for twenty-four hours, melted with decomposition at 186–188° and contained 18.06% nitrogen when analyzed by the Kjeldahl method with mercury catalyst. These values were not altered through four recrystallizations of one sample and did not vary with a change in the recrystallizing solvent. Sulfur and phosphorus could not be detected. No ash remained on ignition.

The recrystallized material was dissolved in water and evaporated on Purina Dog Chow in quantities which would give final concentrations in the dried food of 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0%. These feeds were then fed to 70–80 Gm. albino rats for thirty-five day periods or until the animal was unable to move. All were affected with severity which increased regularly with an increased level. The 0.25% level produced only transitory symptoms. The 0.5% level did not produce severe skeletal changes but those which developed were definite and permanent. The effects with the higher concentrations were severe and equaled or far exceeded those obtained with peas fed at the 70% level (5). There was no apparent difference in toxicity of samples from different preparations.

DISCUSSION

The procedure given has proved to be entirely reproducible and always yields a material which is capable of predictable reproduction of the lathyritic syndrome. The available facts do not allow a claim of purity though we feel further work will justify such a conclusion. Assuming the compound to be pure, the results of feeding trials indicate the concentration in the original pea to be of the order of 0.6–0.8%.

This material appears to be distinctly different from that present in *L. sylvestris Wagneri* and obtained in a concentrated form by Schulert and Lewis. Its alcohol solubility appears to be greater and its toxicity appears to be of a lesser order. The dosages which we have fed are far in excess of those which they (2) report rapidly produce hyperirritability, convulsions, and death. Indeed, we have never observed hyperirritability or convulsions in our animals. Our material seems rather to act as a depressant. It has previously been stressed that *L. sylvestris Wagneri* produces a syndrome unlike that obtained with the Singletary pea (5) or most other *lathyrus* (1, 2). The reasonable conclusion seems to be that the toxicities of these two species cannot be ascribed to a single common toxic compound.

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Notes

A Note on the Thermal Instability of 2-Nitrobutanol-1*

By CLIFTON EUGENE MILLER

IN CONNECTION with our study of nitroalkyl ethyl carbonates (1), we have experienced several explosions during distillation of the intermediate compound 2-nitrobutanol-1. It is the purpose of this communication to report the conditions under which the thermal instability occurred, and as a guide for others, what steps in our opinion should be taken to prevent this hazardous condition.

A commercial sample of 2-nitrobutanol-1 was dried by shaking with anhydrous sodium sulfate, filtered, and distilled. The distillation was interrupted at the end of the day and continued the following morning. It was observed that some thickening of the material in the still pot had occurred overnight. After about two hours of distilling with a take-off temperature of 105°/10 mm. and with the still pot charged to about 35% of the original volume the material in the still pot violently decomposed. The rubber safety plug on the side neck of the two-necked distilling flask was ejected. A heavy carbonaceous material filled the distilling flask, Vigreux column, and condenser. The room was filled with a very lachrymatory smoke but no fire resulted. On another occasion the otherwise clear distillate was observed to be milky just before the explosion occurred.

In view of this thermal instability of 2-nitrobutanol-1 the following conclusions can be drawn: (*a*) distill in quantities small enough to ensure completion of the distillation without interruption; (*b*) do not allow the material in the still pot to diminish to less than about 40 per cent of the original volume taken; (*c*) the use of a two-necked distillation flask, one neck of which is fitted with a rubber blow-out cap is considered essential; (*d*) because of the unknown composition of the gases formed as a result of the decomposition it is recommended that the source of heat be of the Glas-col mantle type, and (*e*) the still operator should be protected by a suitable safety glass shield.

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A Note on the Properties of Some Additional Chloral and α, α, β-Trichlorobutyraldehyde Amides*

By JOSEPH P. LaROCCA† and WOODROW R. BYRUM‡

THE PURPOSE of this paper is to report the synthesis of five new amide derivatives of chloral and α, α, β-trichlorobutyraldehyde and to present the pharmacological data on members of these series not previously reported.

The new compounds were synthesized by the method reported by LaRocca, *et al.* (1), and are listed in Table I.

The pharmacological studies were made as described by Byrum and LaRocca (2). The data obtained in this work substantiate the authors' earlier contention that branching the amide chain enhances the sedative-hypnotic activity (2). In previous work it was shown that chloral 2-ethylbutyramide was more active than chloral caproamide and chloral 2-ethylcaproamide more active than the caprylamide. The present data show that chloral valeramide (HD_{50}—368 mg./Kg.) is less active than the

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† Professor of Pharmacy, School of Pharmacy, University of Georgia.
‡ Director, Department of Pharmacy, Howard College, Birmingham, Alabama.

TABLE I.—CHLORAL AMIDES[a] [$RCONHCH(OH)CCl_3$] AND α, α, β-TRICHLOROBUTYRALDEHYDE AMIDES[a] [$RCONHCH(OH)CCl_2CHClCH_3$]

Compound	Yield, %	M. p., ° C.[b]	Nitrogen[c] Calcd.	Nitrogen[c] Found
Chloral—				
Chloroacetamide	50	145	5.81	5.55
Phenylacetamide	77	143	4.96	4.70
α-Phenylbutyramide	28	133	4.51	4.54
α, α, β-Trichlorobutyraldehyde—				
Isobutyramide	75	134	5.33	5.11
Phenylacetamide	37	107	4.53	4.71

[a] The *Chemical Abstracts* name for these compounds, N-(2,2,2-trichloro-1-hydroxyethyl) and N-(2,2,3-trichloro-1-hydroxy butyl)-amides were not used in this paper because they are unwieldy.
[b] All melting points on calibrated Fisher-Johns apparatus.
[c] Microanalyses by Oakwood Labs., Alexandria, Virginia.

TABLE II.—THE ACUTE TOXICITY AND HYPNOTIC ACTIVITY OF CHLORAL AND α, α, β-TRICHLOROBUTYRALDEHYDE AMIDES ADMINISTERED INTRAPERITONEALLY IN RATS

Compound	Total No. Animals	LD_{50}, Mg./Kg., and Range	HD_{50}, mg./Kg., and Range	Therapeutic Index, LD_{50}/HD_{50}	Av. Duration Narcosis with HD_{50} Min.
Chloral—					
Valeramide	86	880 (677–1044)	368 (271–425)	2.28	62
Phenylacetamide	50	>1200	390 (285–490)	>3.07	52
α, α, β-Trichlorobutyraldehyde—					
Valeramide	20	>1200	1200 (approx.)	>1.00	70
Isovaleramide	63	>1200	682 (502–760)	>1.75	46
2-Ethylbutyramide	68	723 (540–928)	298 (242–371)	2.42	60
Phenylacetamide	59	695 (522–803)	305 (189–490)	1.81	38

previously determined isovaleramide derivative (HD_{50}—275 mg./Kg.). In the α, α, β-trichlorobutyraldehyde series, the isovaleramide and 2-ethylbutyramide derivatives are more active than the corresponding valeramide and caproamide derivatives. In every instance the branched chain derivative was found to be more active than the corresponding straight chain compound. The data are presented in Table II.

These compounds also showed some activity as anticonvulsants when screened for this type of action by Eli Lilly and Company. For this information the authors are indebted to Dr. K. K. Chen.

SUMMARY

1. Five new derivatives of chloral and α, α, β-trichlorobutyraldehyde have been synthesized and characterized.

2. Pharmacological data confirm the postulation that in these compounds branching of the amide chain enhances sedative-hypnotic activity.

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